ENHANCING THE EFFICACY OF DNA VACCINES

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ABSTRACT

Bovine herpesvirus-1 (BoHV-1) causes recurrent respiratory and genital infections in cattle; and infection of cattle with this virus predisposes them to lethal secondary bacterial infections. A primary strategy to prevent and reduce the severity of disease associated with BoHV-1, and to reduce the virus' transmission, is to vaccinate cattle against the virus. Problems associated with the current commercially available vaccines against BoHV-1, which are either modified live (MLV) or killed (KV/inactivated), include that the vaccines are expensive to produce, can cause disease (MLV) or may be ineffective (KV). Development of an effective, non-viral vaccine for BoHV-1 has the potential to address these shortcomings.

DNA vaccines are non-viral. They are economical to produce, and they are safe. The amount of antigen expressed after DNA immunization is small (picogram to nanogram range) however, and this presents a problem with respect to successful immunization of large animals such as cattle. Since only dendritic cells (DCs) can prime immune responses, it is sensible to think that a more robust response to a DNA vaccine could be initiated by engineering the vaccine, the DNA in the vaccine, or the DNA's expressed antigen in such a way as to attract DCs. One way to achieve this would be to incorporate a peptide that is chemotactic for DCs to bring the DCs to the site of vaccination where the antigen is being produced. Research suggests a role for beta (β)-defensins; peptides that are released by cells in response to injury or infection, in attracting immature DCs (iDCs) to the site of vaccination and the subsequent induction of immune responses. Accordingly, I hypothesized that: 1) bovine β-defensins would be chemotactic for bovine iDCs; 2) using a bovine β-defensin in a DNA vaccine could attract iDCs to the site of DNA vaccination and that this attraction would effectively target the DNA-encoded BoHV-1

antigen truncated glycoprotein D (tgD) to the DC; and 3) the enhanced priming by targeted DC would improve humoral and cell-mediated immune responses (CMI) and subsequently protect cattle upon challenge with BoHV-1.

In the first study I characterized the immature state of the bovine DC and then these bovine iDC were used to screen the panel of synthesized bovine β-defensins for chemotactic activity. Previous to this work, neither an iDC nor the chemotactic nature of any of the β-defensins, had been described for cattle; thus both parts of this study were novel in nature. I showed that bovine monocytes (Mo), positively selected from peripheral blood by magnetic-activated cell sorting (MACS), differentiated to iDCs within only 3 days (DC3). Phenotypically, only expression of the mannose receptor (MMR), and functionally, only endocytosis of dextran were defining characteristics of the most immature stage for bovine DCs. Additionally, chemotaxis to the fourteen synthesized bovine β-defensins peptides was almost two-fold higher by the immature DC3 than by day 6 monocyte-derived-DC (DC6) . Through further studies and analysis of the DC3 chemotaxis data I discovered and confirmed that bovine neutrophil β-defensin (BNBD) 3, BNBD9 and enteric β-defensin (EBD) were equally the most chemotactic of the fourteen synthesized peptides. Because BNBD3 was highly chemotactic for iDC and since it is also the most abundant of the thirteen BNBDs in the bovine neutrophil, I chose BNBD3 as the peptide I would use for the rest of the project. To address concerns regarding the correct folding and fold-related functionality of the synthesized peptides, I compared three synthesized variants of BNBD3 with native BNBD3 (nBNBD3) using comparative high-performance liquid chromatography (HPLC) and iDC chemotaxis. I showed that all three synthesized BNBDs were equally chemotactic as the nBNBD3 for bovine iDCs. Since this functionality is fold-related, the

chemotactic equivalency observed strongly supported the conclusion that the BNBD3 synthesized peptides were correctly folded and by extension, that all synthesized peptides used in this study were correctly folded. Lastly, the synthesized analog BNBD3 (aBNBD3), was tested for *in vivo* iDC chemotactic activity by injecting it into the skin of cattle. Cluster of differentiation (CD) 1^+ DCs infiltrated in response to aBNBD3 injection proving that BNBD3 could attract bovine iDCs in the skin.

In the second study, I constructed plasmids that expressed the selected peptide, BNBD3; either alone or as an N-terminal fusion construct with the BoHV-1 antigen tgD, and then tested the effects of the plasmids as vaccines in both mice and cattle. pMASIA-tgD, pMASIA-BNBD3 and pMASIA-BNBD3-tgD were successfully constructed with all plasmids correctly expressing their respectively encoded genes. In mice, inclusion of BNBD3 on a separate plasmid (pMASIA-BNBD3 + pMASIA-tgD) improved cell-mediated immunity (CMI) but did not improve humoral response. Inclusion of BNBD3 in the DNA vaccine as a fusion construct (pMASIA-BNBD3-tgD) was unable to improve the humoral response and did not increase but rather modulated CMI through enhanced induction of tgD-specific cytotoxic T lymphocytes (CTLs) and induction of an interesting population of $CD3^- CD8^+$ interferon gamma $(IFN\text{-}\gamma)^+$ cells that may have been splenic DCs.

In cattle, BNBD3 delivered as a separate plasmid was unable to improve either CMI or humoral responses, whereas inclusion of BNBD3 in the DNA vaccine as a fusion construct improved CMI but did not improve humoral response. After challenge with BoHV-1, the addition of BNBD3 to tgD as a fusion construct vaccine improved the CMI response as observed in

increased magnitude of the IFN-γ response, improved induction of tgD-specific $CD8^+$ T cells, and increased proportion of $CD25^+$ CTLs in the $CD8^+$ T cell subset; yet was unable to improve the humoral response, although the immunoglobulin (Ig) G and virus neutralizing (VN) antibody responses were maintained. Clinically, cattle vaccinated with either pMASIA-BNBD3-tgD or pMASIA-tgD showed comparable reduction in virus shedding, rectal temperature and weight loss. Thus both vaccines were equally protective. Given that humoral immunity was not enhanced, and that inefficient humoral immune responses have been implicated in a lack of protection from BoHV-1 challenge, these results suggested that the humoral immune responses were not high enough and that the improved cellular immunity induced by BNBD3 was not sufficient to result in enhanced protection from BoHV-1. Thus successful DNA vaccination strategies for BoHV-1 will likely need to improve the humoral response while maintaining strong cellular immunity.

The third study was designed to assess an alternate strategy to improve humoral responses to pMASIA-tgD by utilizing BNBD3 in its peptide form, as a complex with the DNA vaccine. The rationale for this work came from the discovery that when a small cationic peptide fused to a short antigenic epitope was complexed at a low peptide to DNA ratio (125:1) with a DNA vaccine encoding for a full length antigen, the humoral immune response to the DNA-encoded antigen could be improved without loss of CMI responses. After establishing bio-activity of the synthesized peptide aBNBD3, I investigated whether vaccine complexes of the positively charged aBNBD3 peptide and the negatively charged pMASIA-tgD DNA could enhance humoral responses of mice to tgD encoded by pMASIA-tgD. Although Low and High peptide to DNA ratio vaccines were also evaluated, only the vaccine consisting of 0.1875 nmol aBNBD3,

complexed with 5 μg (0.0015 nmol) of pMASIA-tgD at the Medium nanomolar peptide to DNA ratio of 125:1 increased humoral responses of mice. CMI was not only maintained relative to pMASIA-tgD, but was modulated to more of a Th1-type response as evidenced by induction of IFN-γ^+ cells and antibody of the IgG2a isotype. To discern the potential mechanism of the complexed vaccine I then examined the effect of BNBD3 on maturation/activation of mouse bone marrow derived dendritic cells (BMDCs). BNBD3 induced phenotypic and functional maturation/activation of *in vitro* treated mouse BMDCs. This is an important aspect of any DC-based vaccine strategy, since after antigen-uptake, the DCs must "mature" in order to traffic (antigen-loaded) from the site of vaccination to the draining lymph node where induction of antigen-specific responses takes place.

With regard to the project hypothesis, I show enhanced efficacy of the humoral responses while maintaining robust cell-mediated responses to a DNA vaccine by the addition of a medium concentration of the synthesized peptide aBNBD3 as a complex with the DNA vaccine; a characteristic of the immune response that was not induced previously by vaccination with the DNA vaccine fusion construct encoding BNBD3 with tgD. I saw induction of IFN-γ secreting cells and an increase in IgG2a antibody production, both of which are desirable. Since both robust antibody and CMI responses of a Th1-type are desired for protection from BoHV-1 infection, and this strategy does result in both, the results of this study are supportive of our project hypothesis and indicate a future direction for a DNA vaccination strategy using complexed vaccines that might be more effective to protect cattle from challenge with BoHV-1.

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DEDICATION

This work is dedicated to my father,

Patrick Thomas Mackenzie.

Brilliant of mind; a philosopher, friend and dad extraordinaire.

Gone too soon.

Missed dearly.

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aBNBD3	Analog BNBD3
AMPs	Antimicrobial peptides
aMΦs	Alveolar macrophages
ANOVA	Analysis of variance
APCs	Antigen presenting cells
β-defensin	Beta-defensin
bICP0	Bovine infected-cell protein 0
BMDCs	Bone marrow derived dendritic cells
BNBD	Bovine neutrophil beta defensin
BoHV-1	Bovine herpesvirus 1
BPIV3	Bovine parainfluenza virus type 3
BRD	Bovine respiratory disease
BRDC	Bovine respiratory disease complex
BRSV	Bovine respiratory syncytial virus
BSA	Bovine serum albumin
BVDV	Bovine viral diarrhea virus
CCL	Chemokine ligand
CCR	Chemokine receptor
CD	Cluster of differentiation
cDCs	Conventional dendritic cells
CI	Chemotactic index
CLP	Common lymphoid progenitor
CMI	Cell mediated immunity
CMP	Common myeloid progenitor
CPE	Cytopathic effect
CpG ODN	CpG oligonucleotides
cRPMI	Complete RPMI
CTLs	Cytotoxic T lymphocytes
CXCL	Chemokine (C-X-C motif) ligand

DC	Dendritic cell
DC3	Day 3 monocyte-derived-dendritic cells
DC6	Day 6 monocyte-derived-dendritic cells
DC-LAMP	DC-Lysosomal-Associated Membrane Protein
ddH20	Double distilled water
E	Early
EBD	Enteric beta defensin
EHV	Equine herpesvirus
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot assay
EMSA	Electrophoretic mobility shift assay
FACs	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
Fc	Fragment, crystalizable
FSC	Forward scattered light
G	Glycine
gD	Glycoprotein D
GM-CSF	Granulocyte-macrophage colony-stimulating factor
hBDs	Human beta defensins
HBV	Hepatitis B virus
HCMV	Human cytomegalovirus
HDPs	Host defence peptides
HHV	Human herpesvirus
HIV	Human immunodeficiency virus
HNPs	Human neutrophil peptides/ human alpha defensins
HPLC	High-performance liquid chromatography
HSV	Herpes simplexvirus
IBP	Infectious balanoposthitis
IBR	Infectious bovine rhinotracheitis
ID	Intradermal
iDC	Immature dendritic cell

IE	Immediate early
IFNα	Interferon alpha
IFNβ	Interferon beta
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IL-4	Interleukin 4
IM	Intramuscular
IPV	Infectious pustular vulvovaginitis
IR	Internal inverted repeat
KV	Killed/inactivated Vaccine
L	Late
LAP	Lingual antimicrobial peptide
LB	Luria-Bertani
LC-DCs	Lagerhans cell-like dendritic cells
LN	Lymph node
LPS	Lipopolysaccharide
mab/mAb	Monoclonal antibody
MACs	Magnetic-activated cell sorting
MALDI	Matrix-assisted laser desorption/ionization
mBDs	Murine beta defensins
mfi/MFI	Mean fluorescent intensity
MHC	Major histocompatibility
MLR	Mixed leukocyte reaction
MLV	Modified live vaccine
MMR	Mannose receptor
Mo	Monocytes
MΦs	macrophages
nBNBD3	Native BNBD3
NEC	Nuclear envelope complex
NKs	Natural killer cells

ORF	Open reading frame
p.i.	Post infection
PAMPs	Pathogen associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
pDCs	Plasmacytoid dendritic cells
pE-BNBD3	Pyroglutamic acid N-terminus modification BNBD3
PM	Proliferation medium
PRRs	Pathogen-recognition receptors
PRV/PrV	Pseudorabies virus (also SuHV)
PTX	Pertussis toxin
Q	Glutamine
R	Arginine
RT/rt	Room temperature
sBNBD3	N-terminus unmodified synthesized BNBD3
SI	Stimulation index
SSC	Side scattered light
SuHV	Suid herpesvirus (also PRV/PrV)
TAP	Tracheal antimicrobial peptide
tgD	Truncated glycoprotein D
TGN	trans-Golgi network
Th	T helper
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TR	Terminal inverted repeat
U_L	Unique long
U_S	Unique short
vhs	Viral host shut-off protein
VN	Virus neutralization
VP	Viral protein
VZV	Varicella-zoster virus (also HHV-3)

1. INTRODUCTION AND LITERATURE REVIEW

1.1 Herpesviruses

Herpesviruses are found in a diversity of species, from mammals to fish to oysters [1]. These viruses tend to be species-specific, which is surprising given the similarities in their structural [2], biological and infectious characteristics [1]. To be classified as a member of the family *Herpesviridae*, these viruses must share a common architecture. In general, they are large (from 120 to 260 nm), complex, enveloped, double-stranded DNA viruses that replicate in the nucleus of their host cells. The mature virion has at its' centre a core that contains the linear double-stranded viral DNA. Surrounding the core is the symmetrical icosohedral viral capsid. Wrapping the capsid is an asymmetrical, historically less well defined area called the tegument [3]. The tegument contains numerous viral proteins that effectively manage the newly infected host cell environment to the benefit of the virus [4, 5]. Finally, on the exterior of the virion is the envelope; a layer that is made up of mostly of host-cell membrane and with viral glycoproteins embedded [1]. Perhaps the most defining characteristic of all the herpesviruses, and certainly the most challenging from the perspective of the vaccinologist, is their ability to evade the clearing action of their hosts' immune system by their ability to persist in the latent state in their natural host. The *Herpesviridae* family is further divided into the sub-families *Alphaherpesvirinae, Gammaherpesvirinae,* and *Betaherpesvirinae.*

1.1.1 Alphaherpesviruses

Alphaherpesviruses are commonplace and pervasive pathogens with a broad host range, a relatively short reproductive cycle, and a lytic action on primary infected cells [1]. Three of the four genera of *Alphaherpesvirinae* (*Simplexviruses*, *Varicelloviruses* and *Iltoviruses*) are neuroinvasive meaning that the virus can invade or spread to cells (sensory/peripheral neurons) that were *not* directly exposed to virus inoculation with the end result of a lifelong latent infection [6]. Although these viruses can infect cells *in vitro* from a wide variety of species, latent infections are typically established in and limited to, the natural host [7, 8]. In humans, two common alphaherpesviruses, namely herpes simplexvirus (HSV/HHV) -1 and HSV-2, of the genus *Simplexvirus*, currently infect more than two-thirds of the world's population [9, 10]. The other common alphaherpesvirus that infects humans is Varicella-zoster virus (VZV/HHV3), of the genus *Varicellovirus* [11]. VZV is the causative agent of chickenpox as the primary infection and of shingles as the reactivation of the latent infection [12]. Equine herpes virus 1 and 4 (EHV-1, -4) in horses, pseudorabies (PRV/SuHV-1) in pigs, and bovine herpesvirus 1 (BoHV-1) in cattle, are important veterinary alphaherpesviruses, and they also belong to the genus *Varicellovirus* [11, 13]. Other ruminant alphaherpesviruses that are closely related to BoHV-1 by way of common antigenic properties and serological relationships, and for which there exists some risk of cross-species infection to cattle, include bubaline herpesvirus 1 (water buffalo), caprine herpesvirus 1 (goat), cervid herpesviruses 1 and 2 (deer), and elk herpesvirus 1 [14].

1.1.1.1 Classification of Alphaherpesviruses

The order *Herpesvirales* contains three subfamilies:the *Alloherpesviridae,* the *Malacoherpesviridae* and finally the *Herpesviridae* (Table 1.1). The family *Herpesviridae* is the largest with 90 formal species sharing common features and is composed of three subfamilies: the *beta-*, *gamma-* and *alpha- herpesvirinae*. Besides common morphological properties, viruses in this family also share several biological properties such as a lytic primary infection followed by the establishment of latency in a specific cell type(s) and thus lifelong latent infections in their natural host. These characteristics have historically formed the basis for classification [15, 16]. The *alphaherpesvirinae* are neurotropic viruses, in that they establish latency in specific neuronal cell populations. *Beta-* and *gammaherpesvirinae* establish latency in lymphocytes and consequently persist in lymphoid organs. *Gammaherpesvirinae* establish early latency, often with little lytic infection and tend to be associated with non-lymphoid cancers and/or lymphoproliferative diseases [15].

As new methods have become available, studies using immunological methods to detect similarities through antigenic relationships, and using molecular methods such as nucleic acid sequencing to reveal genetic similarities have allowed for further refining of the classification into genera [16]. Classification of the *alphaherpesvirinae* subfamily was recently updated in 2012 by the *International Committee on Taxonomy of Viruses* [17] such that it now has five assigned genera that include *Iltovirus*, *Mardivirus, Scutavirus Simplexvirus and Varicellovirus*. Within the genus *Varicellovirus* are found VZV (human natural host), PRV (swine), EHV-1 and 2 (equine), CvHV-1and 2 (deer), CpHV-1 (goat), BuHV-1 (water buffalo), and BoHV-1 (cattle).

Table 1.1 Classification and nomenclature of alphaherpesviruses

Order	Family	Subfamily	Genus	Species (natural host)	Informal name
Herpesvirales	*Malacoherpesviridae*				
	Alloherpesviridae				
	Herpesviridae	*Gammaherpesvirinae*			
		Betaherpesvirinae			
		Alphaherpesvirinae	*Iltovirus*	*Gallid herpesvirus 1* (chickens)	Infectious laryngo-tracheitis virus
			Mardivirus	*Gallid herpesvirus 2*	Marek disease HV 1
				Gallid herpesvirus 3	Marek disease HV 2
			Simplexvirus	***Human herpesvirus 1***	**HSV-1**
				Human herpesvirus 2	HSV-2
			Varicellovirus	*Bovine herpesvirus 1* (cattle)	IBR/IPV
				Bubaline herpesvirus 1 (water buffalo)	
				Caprine herpesvirus 1 (goat)	
				Cervid herpesvirus 1 (deer)	
				Cervid herpesvirus 2	
				Elk herpesvirus 1 [a]	Elk HV [b]
				Equine herpesvirus 1 (horses)	
				Equine herpesvirus 4	
				Human herpesvirus 3	**VZV**
				Suid herpesvirus 1 (pigs)	PRV

(Adapted from Pellett and Roizman (2007) [15] and Pellet and Roizman (2013) [18]). The naming of elk herpesvirus as [a]elk herpesvirus 1 from Thiry et al. (2006) [14] and as [b]ElkHV from Deregt et al. (2000) [19]. BoHV-1 classification is shown by grey areas and the reference species are in bold.

1.1.1.2 Virion Structure

Much of what is known regarding the structure of the alphaherpesvirus virion has been drawn from studies of the prototypical alphaherpesvirus, HSV-1. As described above for herpesviruses, alphaherpesvirus virions are composed of four structural elements, a core containing the double-stranded DNA genome, an icosohedral capsid around the core, the tegument that surrounds the capsid, and the viral envelope containing viral membrane glycoproteins [20]. The core of a typical herpesvirus is composed of a single molecule of viral nonchromatinized linear dsDNA wrapped around a fibrous torus (donut)-shaped, spool-like core [21, 22]. The torus of some herpesvirions appears suspended by fibrils passing through the center of the spool and attaching to the inner side of the capsid [18]. In HSV-1, the DNA is known to be packed tightly and effectively pressurized to facilitate injection of the viral genome into the nucleus of the host cells [10], and a small amount of viral DNA may exist in circular form [23].

Around the core is the capsid which is an 100-nm T=16 icosahedron composed of many copies of four conserved capsid proteins viral protein (VP) 5 / unique long (U_L) 19, VP26/U_L35, VP23/U_L18, VP19C/U_L38) [23], that with the U_L6 gene product, make up a portal complex (through which the viral genomic DNA is loaded into or released from the capsid), 150 hexomeric (faces and edges) and 11 pentametric (vertices) capsomeres connected in groups of three [23, 24]. Studies with HSV-1 show that the portal complex is created with a dodecameric ring of U_L6 encoded protein at one vertex of the capsid and the portal capping protein of the mature capsid is formed by the U_L25 gene product [25]. These two proteins are considered capsid proteins and are products of genes conserved among the herpesviruses [18]. Lytically infected cells harbour capsids in three forms that represent stable intermediates or end products

of herpesvirus capsid assembly: capsids with no core structure (A capsids); capsids with an assembly scaffold but no genome (B capsids); and capsids that contain the genomic DNA core, no scaffold proteins and a portal capping protein (C capsids) [18, 24].

Surrounding the capsid is the tegument which makes up approximately 40% of the virion protein mass [26], and which has been described as an amorphous proteinaceous layer of varying thickness [18]. The tegument is an ordered structure made up of more than 20 different virally encoded proteins; some of which are acquired in the nucleus and make up the inner tegument, and others located at the periphery that interact with envelope glycoproteins [18]. The tegument proteins provide the virus with a supply of pre-made proteins that can manipulate the host cell environment to assist in both viral replication/propagation as seen with the transcriptional activator VP16 of HSV-1 [24] and the U_L82 encoded transcriptional activator of human cytomegalovirus (HCMV/HHV5) pp71 [27]; and in evasion of host cell defences as observed by the action of the HSV-1 virion host shut-off protein (vhs) U_L41 that degrades host cell mRNA [28] and the HCMV U_L83 encoded pp65 protein that blocks major histocompatibility class I molecule (MHC I) presentation of viral proteins [29] and inhibits induction of host interferon (IFN) responses [30].

The outermost layer of the virion is the viral envelope that has been described as a lipid bilayer [31] with a trilaminar appearance [18]. The envelope is compositionally similar to the organelle and cellular membranes through which the viral particle has passed on its way out of the infected host cell and thus consists of lipids, proteins (host cell and viral) and viral glycoproteins that are embedded in the envelope and protrude in a spike-like manner [18, 24]. Importantly, some of

these viral glycoproteins are involved in, and essential for, viral entry into the host cells [32]. Among herpesviruses the number of glycoproteins encoded varies, few are conserved with the exceptions of gB and gH/gL [31], and not all that are encoded are actually found present in the virion [24]. In HSV-1, 20 glycoproteins are encoded and 11 are found present in the virion [33]. Envelope glycoproteins of the HSV virions include: gB (VP7 and VP8.5, encoded by the U_L27 gene), gC (VP8, U_L44), gD (VP17 and VP19, unique short (U_S6)), gE (VP12.3 and VP12.6, U_S8), gG (U_S4), gH (U_L22), gI (U_S7), gK (U_L53), gL (U_L1), and gM (U_L11) (reviewed in [23]). For the alphaherpesviruses HSV-1, PRV and BoHV-1, binding of virus to cells is primarily by gC [34], and for entry and infection of cells gB, gD, gH and gL are required [34, 35]. Thus gB, gC, gD, gH and gL are targets for adaptive immunity, and in HSV they may also be involved in initiating the early innate immune response to the virus [35].

Glycoprotein D (gD) is a type I membrane glycoprotein that is involved in virus entry into the host cell by its interaction with one of several potential entry receptors in the case of HSV-1 (reviewed in [35]), or only nectin-1 (poliovirus receptor-related protein 1) and poliovirus receptor (Pvr) in the case of BoHV-1 [36]. Cellular expression of gD may inhibit subsequent infection by BoHV-1 as has been described by Dasika and Letchworth (1999) for gD of homologous BoHV-1 strain [37]. Superinfection, defined as "infection occurring after or on top of an earlier infection" [38, 39], is of concern in vaccine development for several reasons; one because the protective response to the first infection may lessen protection against subsequent infection by the superinfecting virus [40], and two because of the risk of recombination between a vaccination strain (ie marker vaccine or MLV) and a field strain [41]. Recombination can occur between different strains of the same alphaherpesvirus species when 1) two viruses infect the

same cell at the same time (co-infection) [42], or 2) two viruses infect the same cell as consecutive infections as would be more likely under natural field conditions, and when the two viruses have common target cells [41]. In a comparative *in vitro* study of full length versus truncated gD, it was found that homologous and heterologous superinfection was inhibited by expression of full-length gD, but bovine cells that expressed the truncated, soluble and secreted form tgD were fully susceptible to infection by BoHV-1 [43]. These findings provide support to the hypothesis of a common gD receptor as interference against heterologous viruses suggests a common receptor pathway for entry [43]. The significance of these findings on the protective ability of tgD in a BoHV-1 vaccine regimen in the face of field challenge is unclear. Meurens et al (2004) importantly point out that all these observations were carried out *in vitro* with gD- or tgD-expressing cell lines and not in situations of natural superinfection; thus it may not possible to attribute the interference of superinfection to that of gD expression by the lytically infected cells [41]. Most significantly to practical purposes, when comparing gD and tgD as DNA vaccine antigens, cattle immunized intradermally with a vaccine encoding tgD were afforded protection against BoHV-1 challenge whereas the cattle immunized with the gD encoding vaccine were not [44]. Thus it does not appear that transfection of cells by either plasmid had any effect on superinfection or susceptibility to infection by BoHV-1, even if the plasmid vaccine and BoHV-1 virus were found to target the same cells.

Glycoprotein D is particularly interesting with respect to development of adaptive immunity and vaccine design. Findings from studies with HSV-1 show that gD is a major component of the virion envelope and also stimulates the production of high titers of neutralizing antibodies [45], therefore it is an attractive vaccine antigen candidate. Although the presence of BoHV-1 gD in

wild-type virus was found to induce apoptotic cell death in PBMCs, affinity-purified gD (up to 5µg/ml) did not induce apoptosis [46]. Since gD alone without other virion components does not activate the apoptotic process, gD and tgD should be able to be used safely as a vaccine antigen.

1.1.1.3 Virion Replication

The replication cycle of the alphaherpesviruses has been mostly determined through studies of HSV, and in particular HSV-1, as the prototypical alphaherpesvirus. Figure 1.1 shows the HSV-1 replication cycle. As such, primary infection of the host normally begins with entry of the

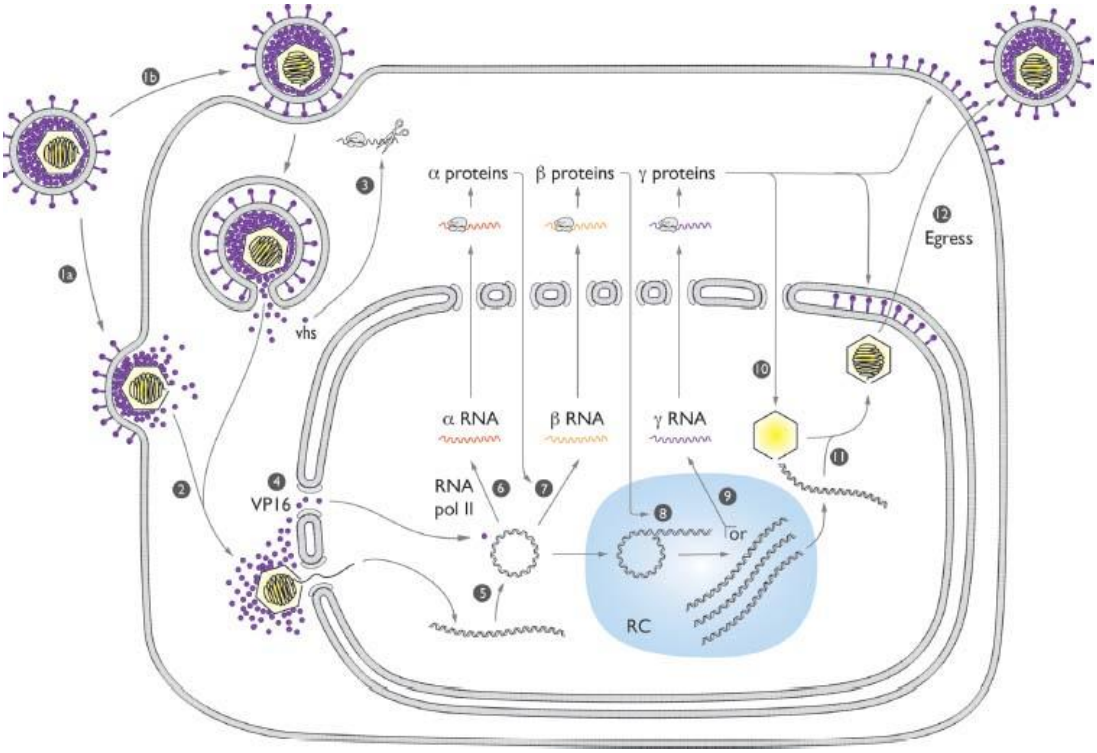

Figure 1.1 Replication cycle of herpes simplexvirus 1.

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virion into the host which occurs typically by inhalation of virus-laden droplets and then engagement/attachment of the virus to epithelial cells of the respiratory mucosa. Next, the virus tethers to the host cell by binding to cell surface molecules of heparin sulphate proteoglycans [23, 35]. Tethering/binding of HSV-1, PRV and BoHV-1 is typically mediated by the viral envelope glycoprotein gC, although this function can also be carried out by gB or gD, both of which also contribute to stable binding [34]. Following tethering, entry can occur by cell-type dependent endocytosis (reviewed in [23]), or by fusion. Fusion of the virion envelope with the host cell plasma membrane is mediated by engagement of the viral glycoproteins gB, gD, gH and gL with their receptors. Specifically, engagement of gD triggers a conformational change that results in recruitment and heterodimerization of gH/gL and activation of gB that enables fusion [23, 34, 35]. Fusion results in the release of the viral nucleocapsid (capsid and tegument proteins) into the cytoplasm. Endocytosed virus that escapes from the endosome is also released as a nucleocapsid to the cytoplasm. Once in the cytoplasm, the capsids follow a network of microtubules that facilitate their movement to the nuclear envelope where they bind to nuclear pores and then release their viral DNA into the nucleus leaving the tegument proteins and empty capsids behind in the cytoplasm [4, 23].

The tegument vhs protein causes degradation of host messenger RNAs in the cytoplasm and the alpha trans-inducing protein VP16 (U_L48) moves from the cytoplasm into the nucleus where, upon circularization of the viral DNA, VP16 facilitates transcription of the immediate-early (IE) or α-genes by host RNA polymerase II to give alpha(α)-mRNAs. Translation of mRNA occurs in the cytoplasm and resultant proteins are either transported back to the nucleus, or to cellular organelle membranes, or they remain in the cytoplasm. Translation of the α-mRNAs results in

six IE proteins, five of which act to regulate viral gene expression in the nucleus including inhibition of further transcription of α-mRNA [23]. Interestingly, cholesterol as a component of lipid rafts is essential in early replication and particularly for virus entry into the host cells for a number of alphaherpesviruses, including BoHV-1 [47], HSV-1 [48], VZV [49] and PRV [50]. This IE phase occurs from about 2-5 hours (h) post-infection (p.i.) [51]. Next, IE proteins transactivate beta (β) gene transcription. Translated β-mRNA early (E) proteins, including the viral DNA polymerase, are involved in replicating the viral DNA and in stimulating gamma (γ) gene expression. Replication of viral DNA signals the end of the early phase (about 5 h p.i.) and the beginning of the late phase of infection [51]. Translated γ-mRNA late (L) proteins include the structural capsid proteins and the envelope glycoproteins. Late proteins are involved in assembling the capsid in the nucleus and in modifying the host cellular membranes for the final step from particle to fully enveloped infectious virion during virus egress [4]. Thus from 5 h p.i. until total cell lysis at 10-20 h p.i., the late phase is dominated by viral DNA replication, synthesis of structural proteins and assembly of viral particles [51].

Capsid assembly takes place in the nucleus where the late proteins form capsomeres that are assembled into capsids, and that process and package the viral DNA into the capsid [4, 23]. The first step in nuclear egress is primary envelopment, whereby capsids are wrapped in the inner nuclear membrane. In HSV [4] and PRV [52], this requires that capsids obtain a nuclear envelopment complex (NEC) containing the viral proteins pU_L31 (nuclear phosphoprotein) and pU_L34 (type II integral membrane protein). In HSV, pU_L31 and pU_L34 also promote the recruitment of pU_S3, a multifunctional serine-threonine protein kinase that, in the function of egress of capsids from the nucleus, serves to alter the structure of the nuclear membranes to

enable budding of capsids into [23], and out of [53], the perinuclear space [23]. Capsids acquire a subset of tegument proteins (VP1-2/pU_L36, pU_L37, vhs, VP22, VP16); and then bud from the nucleus to the cytoplasm utilizing a process that preferentially selects for C-capsids and requires a nuclear envelopment (NEC) complex around the capsid. Capsids progress through membranes of cytosolic structures by sequential envelopment and de-envelopment. Tegument proteins are added at several locations throughout the process of egress and viral envelope glycoproteins are added during secondary envelopment in the *trans*-Golgi network (TGN) [4, 23]. Interestingly, a few of the glycoproteins of HSV (gJ, gK, and gN) that are rarely recruited to the sites of secondary envelopment are also rarely found in extracellular virions [54]. This suggests some importance to the process of selection and sorting of viral glycoproteins by the secondary envelopment sites. Lastly, enveloped virions within TGN-derived membrane vesicles are exocytosed by the cell following fusion of the TGN-derived membrane to the host cell plasma membrane and most enveloped virus particles are found attached to the outer surface of the cell plasma membrane as opposed to being fully released from the cell. This puts the infectious virions in close contact with adjacent cells and encourages cell-to-cell spread [4, 23].

1.1.2 Bovine Herpesvirus

Bovine herpesvirus-1 (BoHV-1) is classified as an alphaherpesvirus, genus Varicellovirus, and is considered a serious pathogen in cattle [55]. BoHV-1 causes recurrent respiratory and genital infections in cattle, and predisposes to lethal secondary infections [56]. Like other alphaherpesviruses BoHV-1 causes not only initial, but recurring disease due to the virus' ability to establish latency [57].

1.1.2.1 Molecular Aspects of BoHV1

As mentioned above, the BoHV-1 genome is a double-stranded linear DNA molecule that is relatively long at 135-136.9-140 kilobase pairs (kbp) [18, 58-61] and has a relatively high guanine and cytosine content of 72% [18]. The BoHV-1 genome structure is that of a D-class herpesvirus, characteristic of the genus Varicellovirus, a structure that is also demonstrated in PRV, VSV and EHV-1 and 4 [18, 58]. As such it is comprised of a unique long segment U_L (103 kbp), an internal inverted repeat I_R (11 kbp), a unique short segment U_S (10 kbp), and a terminal inverted repeat T_R (11 kbp) [62]. DNA replication begins following formation of a circular molecule as a result of ligation of the termini of the linear genome. Replication by rolling circle model from this circular DNA molecule results in recombinational events [58] and the accumulation of many concatemers (complex concatemeric intermediates), which are multiple copies of the genome linked end-to-end, in the cells [58, 62, 63]. At the time of DNA replication (in the concatemer) the U_S segment inverts relative to the U_L segment resulting in two isomers where the U_L segment is fixed and the U_S is in either of two orientations called the prototype (P) orientation [58, 62]. The U_L segment can also invert, giving rise to four isomers, although genomes with this inverted U_L segment (I_L orientation) are only found within the concatemeric DNA. Cleavage of DNA concatemers favors the two isomers with the P orientation and this results in predominantly equimolar amounts of two isomers of the linear double-stranded DNA molecule packaged into virions [58, 62]. The 73 open reading frames encoded by the BoHV-1 genome are homologous to genes of other alphaherpesviruses and are thus labelled relative to those of HSV-1 with the exception of the BoHV-1-specific gene, $U_L0.5$, and a few other genes specific to BoHV-1 [61] or to varicelloviruses. These genes and their corresponding proteins are reviewed very nicely in table form comparatively with the genome of BoHV-5 [64] and also in

relation to the timing of their production in the viral life cycle (immediate early, IE; early, E; late, L) and their essentiality with regard to *in vitro* viral replication [65]. The U_L region of the genome encodes proteins involved in viral replication and processing, and also proteins that make up the capsid, tegument, and six envelope glycoproteins, gK (U_L53), gC (U_L44), gB (U_L27), gH (U_L22), gM (U_L10), and gL (U_L1) [61, 64]. The gene that encodes gN, U_L49.5, is also within the U_L region [64], but as this protein is not glycosylated in BoHV-1, it is considered a "false protein" even though it functions in a conserved manner by forming a disulfide-linked heteroduplex with gM (reviewed in [59]). The U_S region similarly encodes both non-structural and structural proteins including the remaining four envelope glycoproteins, gG (U_S4), gD (U_S6), gI (U_S7), and gE (U_S8) [61, 64].

1.1.2.2 Infectious Nature of BoHV-1 and Immune Responses to BoHV-1 Infection

1.1.2.2.1 BoHV-1 Primary Infection

BoHV-1 has evolved strategies to evade clearance and ensure survival that involve modulation of itself and modulation of the host immune system. BoHV-1 modulates host responses by transiently suppressing the immune system in a number of ways that support viral infection, often through impairment of the normal functioning of the immune system. The first way is by compromising the **first line of defence**, the integrity of the respiratory tract. The respiratory form of BoHV-1 infects epithelial cells of the upper respiratory tract. Lytic replication ensues where infected epithelial cells increase in size, develop intranuclear inclusions, and ultimately necrotize or undergo apoptosis (releasing large numbers of progeny virus) in what is known as the cytopathic effect (CPE) of BoHV-1 lytic infection [59]. As well as direct CPE, BoHV-1 interferes with repair of the airway epithelium by inhibiting the migration of new epithelial cells

to injured areas [66]. The high numbers of virus particles that are shed via the nasal mucus, and the fast replication cycle of the virus result in rapid spread of infection within a cattle herd [67]. Damage to the epithelial cells of the upper respiratory tract thus disrupts the first line of defense against bacterial colonization of the lung by impairing ciliary activity with a resulting loss of function of the mucociliary escalator. Resident, commensal bacteria of the nasopharynx (ie. *M. haemolytica)* [68-70], that would normally be coated in mucus and then expelled by the constant upward movement of the epithelial cilia, can then migrate or be inhaled such as to gain access to the lower respiratory tract where they multiply rapidly. BoHV-1 also triggers a bronchoconstriction that further traps secretions and bacteria in the lungs [71]. BoHV-1 can then gain access to the central nervous system by penetrating nerve endings present in the infected epithelia of the upper respiratory tract [72].

The **second line of defense** is the innate or non-specific inflammatory and cellular reactions to BoHV-1 infection. These include (but are not limited to) release of the type I-IFNs (IFNα and β) from infected cells that result in recruitment of alveolar macrophages (aMΦ), neutrophils and large granular lymphocytes (natural killer cells (NKs)). These effector cells in turn release more of these early cytokines in the infected epithelium which attract cells of the innate (DCs) and the adaptive immune system (T helper-cells), including CD8+ CTLs (MHC I-restricted cytotoxic lymphocytes), and activate MΦs and CTLs to kill virus-infected cells [59]. Cattle are able to mount a strong and effective innate response to eliminate virus, but in addition to latency that makes it impossible to completely eliminate the virus, BoHV-1 encodes two proteins that inhibit the innate immune response and favour viral replication [59, 73]. Bovine infected-cell protein 0 (bICP0) increases transcription of viral proteins and inhibits transcription of interferons (IFNs) -

in particular IFNα [59, 73]. The subsequent lower numbers of recruited alveolar MΦs and their severely reduced activity interferes with an important part of lung defense (alveolar MΦ phagocytosis and killing of engulfed bacteria) and favours infection by bacteria. Glycoprotein G binds lymphocyte-attracting chemokines, effectively reducing their numbers at the site of infection that would reduce CTL killing of infected cells and impair development of a robust adaptive immune response [59, 73].

BoHV-1 strategies that interfere with the development of the adaptive immune response impair the **third line of defense**. BoHV-1 infects leukocytes that can spread the virus systemically whereupon it has been suggested that infection of distant organs [59, 74] can occur. As well, infection of lymphocytes causes suppression of their proliferative ability [75, 76]. Infection by BoHV-1 causes CD4+ cells to lose their CD4 molecules which induces apoptosis of the CD4+ T helper cells [77]. Thus the number of CD4+ Th cells is reduced as they apoptose and are effectively removed from the circulation. With reduced numbers, T helper cell function and cytokine production is impaired which in turn lowers humoral and cell mediated responses. Viral protein U_L41.5 (gN) blocks assembly of peptide-MHC I complexes thus inhibiting recognition and subsequent killing of infected cells by CD8+ CTLs [78].

The bacteria implicated in bovine respiratory disease (BRD) subsequent to BoHV-1 infection also have the ability to negatively impact the immune response. *M. haemolytica* produces leukotoxin, an exotoxin that attaches to CD18 expressed on all leukocytes and induces cytolysis, thus reducing the number of both innate and adaptive immune system cells [79]. Leukotoxin also down-regulates expression of MHC II molecules on MΦs and DCs, thus reducing antigen

presentation [80]. The polysaccharide capsules of *M. haemolytica* make the bacteria less susceptible to complement-mediated serum killing, and reduce phagocytosis by neutrophils and MΦs [81]. Thus while BoHV-1 immune evasion strategies seek to increase viral replication and survival, these viral strategies additionally perpetuate an environment favourable for the bacteria; and while the immune modulating activities of the bacteria serve to lessen the potency of the immune response against the bacteria themselves, these strategies result in an environment beneficial to both bacteria and BoHV-1.

Infection with BoHV-1 is responsible for a wide range of disease manifestations that are not limited to respiratory disease of feedlot cattle, but also include respiratory and reproductive disease in dairy and beef breeding herds [82]. Clinical symptoms of the respiratory form, also known as infectious bovine Rhinotracheitis (IBR) include rapid breathing, loss of appetite, fever, coughing, nasal discharge, foamy salivation, open-mouth breathing, severe inflammation of the nasal passages and tissue surrounding the eyes (conjunctivitis), and loss of weight and condition. In calves under 4 months of age, sequelae of infection can include meningitis or fatal systemic disease leading to death. In the breeding herd, cows exposed between 5-7 months gestation may abort, and in dairy animals milk yield may be reduced [83]. Infectious pustular vulvovaginitis (IPV) and Infectious balanoposthitis (IBP) diseases manifest in cows and bulls respectively and the symptoms include fever, genital tract lesions (plaques) and genital discharge. Less frequently, BoHV-1 has also been reported to cause digestive tract disease (enteritis), dermatitis and lesions of the interdigital space, and a catarrhal type of mastitis [84].

1.1.2.2.2 BoHV-1 Latency

BoHV-1 has evolved strategies to evade clearance and ensure survival that involve modulation of itself and modulation of the host immune system. The most important viral modulation, and a characteristic of alphaherpesviruses, is the ability to establish latency. BoHV-1 is thought to gain access to the central nervous system by penetrating nerve endings present in the infected epithelia of the upper respiratory tract [72]. In the absence of BoHV-1 lesions, it is unlikely the virus would have access to these nerve termini. The virus is then transported along the microtubules of the axons to the nucleus in the body of the trigeminal and sacral ganglions where it establishes latency [59]. In the neuron, BoHV-1 transcript expression is modified such that only two proteins are translated and expressed. High levels of the latency related gene product, that serves to turn off viral expression and inhibit apoptosis of the neuron [85], and the open reading frame (ORF) -E gene product, that enhances neuronal health [86] are expressed. Due to their abundance, these gene products are thought to be actively involved in the maintenance of latency [59]. Natural stressors that lead to increased secretion of corticosteroid (or experimental administration of cortisol) and/or immune suppression can initiate reactivation from latency [59]. Once reactivated, there is a reversing of the above-described events with viral gene expression occurring in the sensory/peripheral neurons and infectious virus shed from the nose and eyes [87, 88]. BoHV-1 can be repeatedly reactivated throughout the animal's productive lifetime causing recurrent infection, viral shedding, and spread of the virus to naïve animals [70].

1.1.2.3 Bovine Respiratory Disease

Bovine respiratory disease complex (BRDC) is multifactorial, resulting from interactions among bovine viral and bacterial pathogens at times of stress [70, 71, 89]. Viruses that are present in

feedlot cattle and that have been implicated in BRDC include BoHV-1, bovine respiratory syncytial virus (BRSV), bovine viral diarrhea virus (BVDV) and parinfluenza-3 virus (BPIV3). BoHV-1 is the causative agent of IBR [55, 90]. Infection by BoHV-1 leading to IBR causes a transient immune system suppression that predisposes cattle to secondary bacterial infection characteristic in BRDC [71]. In Canada and the US, the causative bacteria are *Mannheimia haemolytica* and *Mycoplasma bovis* [71, 73]. Other bacteria that infect the bovine respiratory tract of cattle are *Histophilus (Haemophilus) somni* and *Pasteurella multocida* [73, 91]. Secondary bacterial infection by these organisms causes pneumonia and it is the pneumonia subsequent to the viral infection (BRD) that leads to morbidity and death of affected feedlot cattle [90].

BRDC results in animal pain and suffering, and a loss of revenue to the to the feeder cattle industry. Economic losses are due to mortality, cost of treatment, and reduced growth performance resulting from BRDC. Using industry data from the United States feedlot and meat industry, industry economic losses of $3 billion (US) each year (USDA:NASS, Cattle on Feed 2002), and feedlot mortality of 1.4%, with 2/3 of those deaths attributable to BRD [92] have been reported. In Canada, BRD accounted for 66% of mortality in Ontario feedlots between the years 1978-1980 [93], and 10-57% of mortality in a large commercial Western Canadian feedlot between the years 1985-88 [89]. In fact, the economic losses attributed to IBR in the cattle industry worldwide [83], are considered so severe, that IBR eradication programmes utilizing BHV-1 glycoprotein E-negative vaccines (to differentiate immunized versus infected animals) were started more than a decade ago in a number of European countries [94].

It should be noted that not all cases of infection by BoHV-1 (IBR) are complicated by secondary bacterial infection with progression to pneumonia and death. The course of the disease varies among animals and outbreaks, and many animals are able to recover [71, 89]. How well they recover depends on care during illness [93], and ability to clear the virus [95]. A relatively long period of low level immune suppression is seen even after the lesions have resolved [95]. This can translate to long term lower weight gains and poor performance in the feedlot [93]. In unvaccinated feedlot cattle, morbidity is usually high (20-30 and as high as 100%) and mortality rate is low (1% and as high as 10%) [71]. Stresses associated with weaning, mixing of cattle at sale yards and feedlots, and shipping tend to increase the severity of IBR and the likelihood of pneumonic BRD [71, 89]. Mortality data over 4 years from a commercial feedlot study showed a rise in mortality due to BRD 14-16 days after arrival at the feedlot (25^{th} percentile), this peaked at 15-19 days (19-22d median), and gradually decreased out to 29-42 days (75^{th} percentile) [89]. The reported incubation period for development of IBR after BHV-1 exposure varies from 1-6 days after experimental challenge to 10-20 days after new cattle are introduced in a field setting [71]. The timing of incubation to mortality supports the association between BoHV-1 infection, stress (mixing and shipping) and development of BRD. It has been suggested that control of BRD involve a combination of vaccination with improved management practices that minimize stress of cattle upon arrival at the feedlot [59, 70, 93].

1.1.2.4 Control of BoHV-1 Infection

Methods to control BoHV-1 typically involve animal management practices to minimize stress and basic biosecurity measures that minimize opportunities for animal to animal transmission. Beyond these very important considerations, the adoption of specialized biosecurity, vaccination

and/or eradication programs tends to differ by country and continent. For example, in many European countries, eradication programs have been in place, and may include strictly serological identification with culling of infected animals, or may additionally include vaccination of animals with a gE deleted marker vaccine (DIVA vaccine) to differentiate vaccinated from infected animals [59]. Interestingly, only the countries that have practiced culling of infected animals while prohibiting vaccination have achieved BoHV-1/IBR-free status (Austria, Denmark, Finland, Norway, Sweden, and Switzerland). The economic loss of otherwise healthy animals through culling would appear to limit practicality of this approach to only those countries with low incidence at the outset of the eradication program [96] or with low animal numbers or concentrations. In the case of the DIVA strategy, the relatively low sensitivity (70%) of the diagnostic ELISA test for BoHV-1 gE antibody presents both a risk of infected animals going undetected, and an economic cost of unnecessary culling of false positive animals (reviewed in [59]). Additionally, establishment of latency and the potential for BoHV-1 recombinants are biosafety concerns with the MLV gE-deleted DIVA vaccines [59, 97]. For eradication in particular and control in general, additional biosecurity measures are recommended that include ensuring breeding stock, embryos and semen are BoHV-1 free; physical separation of positive and negative animals; and knowledge of transmissibility of BoHV-1 through feed that dictate feedstuff contamination control measures in the feedlot (reviewed in [96]). In North America, relatively high seroprevalence [98], differences in geography, climate, animal numbers and concentrations dictate practices that are, by necessity, quite different. Here also though, it has been suggested that control of BoHV-1/IBR/BRDC involve a combination of vaccination with improved management practices that minimize stress of cattle upon arrival at the feedlot [59, 70, 93]. An area that is often ignored is management of

cattle that are already in the feedlot, past the time of highest risk for IBR. A recently reported increase in IBR outbreaks in vaccinated feeder cattle points to a need for attention to this group. Reversion to virulence of modified live vaccines, or emergence of a new BoHV-1 strain have been suggested as possible reasons for these outbreaks [99, 100]. Other possibilities to consider are changes in the genetics of cattle currently on feed (more Angus than the traditional Hereford/Charolais/Limousine influence) that may not respond as well to vaccine strains or that might require different management. Also, a move to year-round receiving of cattle from many sources into feedlots (this is different than the traditional entry of newly weaned cattle in the fall) means that cattle currently in the feedlot have an increased chance of exposure to pathogens carried by newly received cattle at a time when protective immunity of the vaccine given to resident cattle at the time of entry may be declining. Thus establishment of long-term immunological memory may be an important consideration for BoHV-1 vaccines for North American cattle.

In Canada and the United States, current strategies to control BoHV-1 emphasize and include vaccination with commercial MLV or KV BoHV-1 vaccines. These commercial vaccines do not, however, completely protect cattle from infection with BoHV-1 [101]. Additionally, the safety and effectiveness of these commercial vaccines are still of concern. As mentioned above, eradication programs using deletion marker vaccines fall short of ideal also [102]. Vaccination of neonates, particularly in the face of maternal antibody, is another area of difficulty that might be overcome with improved vaccines. Recently it has been reported that a combination of adjuvant with a BoHV-1 MLV resulted in enhanced immunogenicity and protective effect [103].

Obviously, the shortcomings of current commercial vaccines point to a need for, and drive the development of, a better vaccine for BoHV-1.

1.2 Dendritic Cells

1.2.1 Dendritic Cells in the Induction of Immune Responses

DCs are unique antigen presenting cells (APCs) as they are the only ones that are able to induce primary immune responses and the subsequent establishment of immunological memory [104, 105]. Dendritic cells are also potent initiators of a cell-mediated response [106]. In a MLR, only one DC is needed to turn on 100-3,000 T cells [106], and in mice, ex vivo-activated DCs can prime recipient animals to respond to an antigenic challenge in an antigen-specific manner within a week [107]. DCs can process exogenous antigen for presentation on both MHC I and MHC II molecules, thus inducing both CD8 (CTL) and Th mediated immunity [108]. Non-replicating antigen is limited, in most APCs, to presentation on MHC class II molecules with subsequent presentation to, and activation of, Th cells [106]. DCs, however, are able to transport phagocytosed/endocytosed antigens from the endocytic compartment to the cytosol [109, 110]. This unique characteristic, called "cross-presentation", allows DCs to present exogenous antigen on MHC I molecules, leading to induction of CD8 T-cell immunity [111]. DCs are able, therefore, to simultaneously present exogenous antigen on both MHC I and MHC II molecules. The subsequent immune response after presentation to T cells is balanced, resulting in the generation of antibody, Th and CTL cells. If a BoHV-1 vaccine could be targeted to DCs, then this characteristic of DCs could be exploited to induce effective immunity to viral infection.

1.2.2 Types of Dendritic Cells

Dendritic cells are non-dividing cells [112] whose precursors originate from CD34+ haematopoietic stem cells within the bone marrow [113]. As shown in Figure 1.2, they are a heterogeneous population with respect to phenotype and function but essentially develop along a pathway from a common precursor belonging to one of two lineages [113, 114]. The first lineage is that of the myeloid cells. These include Mo, MΦs, DCs and granulocytes that arise from a common myeloid progenitor (CMP). The second lineage is that of the lymphoid cells including T cells, B cells, NK, and a certain subset of DC that arise from a common lymphoid progenitor (CLP) [115]. There is some question, however, as to whether the plasmacytoid DCs (pDCs) actually do arise from a CLP, or whether they derive from a myeloid origin common DC precursor (CDP) [116], or both [117, 118]. Beyond ontogeny, DCs are grouped as either conventional DCs (cDCs), monocyte-derived DCs (MoDC) or plasmacytoid DCs (pDCs). There is also some debate as to whether the activated MoDCs, termed by some as "inflammatory" DCs, and the activated interstitial cDCs (derived from the CLA^- CDP) are *in vivo* functional equivalents [119]. If so, then the MoDCs would also be grouped as a cDC. DCs are further described as either migratory or lymphoid tissue-resident, based on whether they stay in one place or not. Migratory DCs are present in the blood at low frequencies, and in peripheral tissues and in lymphoid tissues at high frequencies. Upon antigen-uptake, activation and maturation, they migrate to the draining lymph node (LN). In contrast, the lymphoid tissue-resident DCs perform all the same DC functions of antigen-uptake, activation and maturation, but they remain within the lymphoid tissue [120, 121]. The most recent work on DC lineages and DC subtypes aligns mouse and human DCs into four subsets irrespective of their location. In mice, they all derive from a $Lin^-CX3CR1^+$ $CD11b^-$ $CD115^+$ $cKit^+$ $CD135^+$ macrophage-DC precursor (MDP)

that derive from the CMP [116], and the subsets correspond to Xcr1+ cDC, CD11b+ cDC, MoDC, and pDC [122]. Regardless of whether they are migratory or resident, notably, it is the cDCs and the MoDCs [123] that have the ability to prime naïve T cells [117].

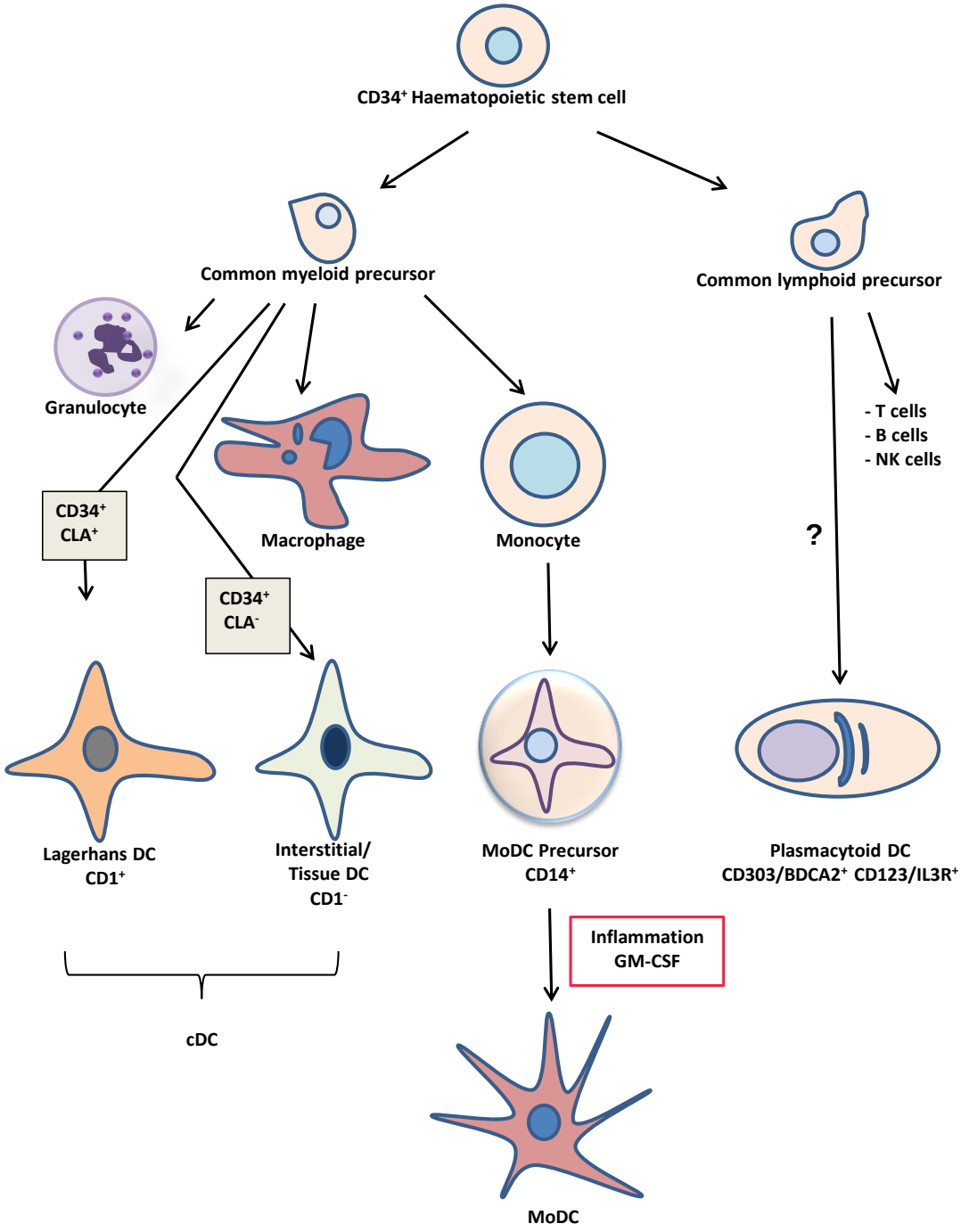

Figure 1.2 DC lineage and development of DC subsets.

CLA, cutaneous lymphocyte antigen; MoDC, monocyte-derived dendritic cell; GM-CSF, granulocyte macrophage colony-stimulating factor

The pDCs are referred to as IFN producing cells or natural IFN producing cells as when activated, they produce massive amounts (10-100 times more than any other type of cell) of type I IFN [124]. pDCs differ from cDCs in that they are found mainly in the blood and are poor antigen presenting cells (APCs). They are, however, considered the main antiviral innate immune response effector cell due to their virus-activated IFN production [125]. In general, cDCs are of the myeloid lineage and pDCs are of the lymphoid lineage [114, 126]. For the remainder, this review will focus on DCs of the myeloid lineage.

1.2.3 Life-cycle of a Dendritic Cell (DC)

1.2.3.1 Immature DCs

Myeloid immature DCs (iDCs) are derived successively from bone-marrow CD34+ progenitor cells and precursor cells as described above. The precursor DCs travel through the bloodstream and migrate to tissues where they reside as immature DCs at body surfaces and interstitial spaces; thus they are typically found in peripheral tissues where they monitor their environment [105, 106, 114]. They can be resident cells such as Langerhans cells of the skin that derive from $CD14^{-}CD11c^{+}$ blood precursors, or they can be infiltrating iDCs or pre-DCs that derive from $CD14^{+}CD11c^{+}$ blood precursors [114]. iDCs are poor stimulators of T cells. iDCs are further characterized by their endocytic ability that allows them to continuously sample from their environment, expression of uptake receptors, abundant MHC II products within intracellular compartments for complexing processed peptide from the antigen that gets taken up [127], expression of chemokine receptors, chemokine receptor (CCR) 1, CCR5 and CCR6 that allow them to selectively home to areas within the tissues of infection and inflammation where chemokines, chemokine ligand (CCL) 3 (MIP-1α), CCL4 (MIP-1β), CCL20 (MIP-3α), and

chemokine (CXCL motif) ligand (CXCL) 10 (IP-10), are secreted in response to pathogens or inflammatory signals from resident tissue cells [128] and from the iDCs [129]. iDCs very capably internalize particles, viruses, bacteria apoptotic and necrotic cell fragments by phagocytosis [130, 131], they sample extracellular fluids and solutes in their immediate environment into large pinocytic vesicles by macropinocytosis [132], and they express adsorptive uptake receptors such as the type-1 transmembrane C-type lectin receptors like the macrophage mannose receptor (MMR/CD206) [132] and DEC-205 (CD205) [133, 134], as well as fragment crystalizable (Fc) γ and Fcε receptors [132]. Thus the immature state of DCs can be identified by functional assessment of proliferative and phagocytic ability and phenotypic assessment for expression of chemokine and uptake receptors.

1.2.3.2 Mature DCs

Maturation of DCs is a continuous process initiated at the site of infection and completed in the LN whereby iDCs undergo phenotypic and functional changes that result in their transition from antigen capturing cells at the periphery to antigen processing and presenting cells at the LN [135, 136]. During maturation (Figure 1.3) expression of CCR1, CCR5 and CCR6 are down-regulated and expression of CCR7 is up-regulated. Expression of CCR7's ligand, SLC (CCL 21) on the endothelium of the afferent lymphatic system, and by the stromal cells in the T-cell zone directs migration of the maturing DC out of the tissue and toward the LN [137]. In the LN, mature antigen-presenting DCs stimulate naïve T-cells through up-regulation of their surface costimulatory molecules CD80 (B7.1), CD86 (B7.2), and CD40, which bind to CD28 and CD40L (CD154) respectively on T-cells. T-cell recognition of the presented antigen leads to T cell-mediated terminal maturation of DCs, a process characterized by further up-regulation

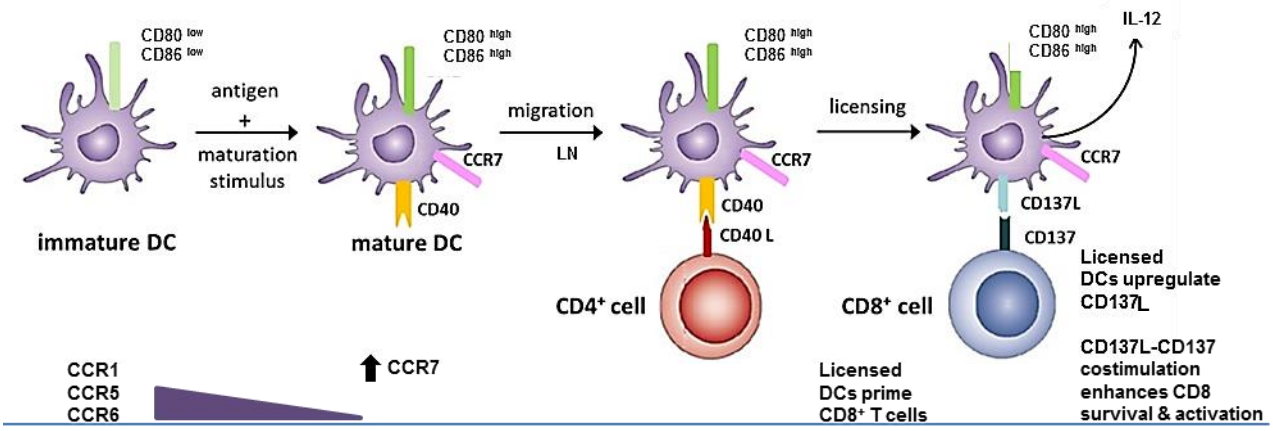

Figure 1.3 Maturation process of DCs. Signals in the environment such as pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPS) activate pattern recognition receptors (PRRs) of iDCs, and along with antigen uptake, initiate the maturation process. Expression of iDC chemokine receptors is down-regulated and expression of the LN homing receptor CCR7 is up-regulated. Maturing DCs migrate to the LN where they present antigen to $CD4^+$ and $CD8^+$ T cells. DCs are activated through CD40:CD40L interaction by the $CD4^+$ T cells which then licence the DCs. Licenced DCs upregulate costimulatory molecules such as CD137L. Priming of CD8+ T cells causes upregulation of CD137 by the CD8+ T cell; and engagement results in CD8+ T cell activation, proliferation, effector function and memory. (Adapted from De Haes et al. (2012) [138]).

of co-stimulatory molecules and the release of IL-1 and IL-12 by DCs and IFN-γ by T-cells [139]. Engagement of CD40 by CD40L of antigen activated CD4 T cells induces mature DC to differentiate further in a step known as "licensing" [138]. Licencing of DCs by CD4+ T cells provides a form of T cell help that amplifies the DCs activity and allows for induction of potent cytotoxic CD8+ T cell responses in the absence of a very strong inflammatory stimulus [140]. Licenced DCs up regulate additional cell-surface molecules such as CD137L, CD70, CD252/OX40L and glucocorticoid-induced TNF receptor-related protein ligand (GITRL). Priming of CD8+ T cells by MHC class I-restricted peptides causes upregulation of the corresponding molecules CD137 [138], CD27, CD134/OX40, and GITR respectively by the CD8+ T cell; and engagement of these molecules with their cognate ligands results in robust

CD8+ T cell activation, proliferation and effector function, as well as the induction and maintenance of memory [141].

Whether T-cells of the Th2 subtype release IL-4 during terminal maturation of DCs, and what effect this would have on terminal maturation of DCs, is currently not clear. However, it has been shown that IL-4 secreted by Th2 cells can strongly enhance the production of bioactive IL-12p70 heterodimer by CD40L-stimulated DC and can synergize with IFN-γ [142]. Theoretically, this increase in IL-12 may amplify the signal to DC to terminally mature; or it may be that the CD40 (DC):CD40L (T-cell) interaction is the critical factor. The presence of B cells and their secretory Ig molecules may also be important for development of fully functional DCs. Splenic cDCs were unable to crosspresent soluble antigen in the absence of T and B cells (lymphopenic mice) and this DC cross presentation was found to be dependent on serum Ig. It was further hypothesized that Ig, either as immune complexes or via Fc receptors, acts through C-type lectin receptors (CD205, CD206/MMR, DC-SIGN) expressed by the DCs [143]. This idea that terminal maturation, given the presence of other activating factors, occurs as a result of CD40:CD40L interaction between the DC and the T-cell is supported by the results of another study whereby cell-free supernatants from activated T-cells that contained sCD40L and IFN-γ could induce terminal differentiation of DC [144]. Thus it may be through the action of CD40 ligation, in combination with the bioactive IL-12p70 heterodimer and IFN-γ synergy, that Th2 or Th1 cells mediate terminal maturation of DCs.

1.2.4 Implications of Infection on DC Function and Subsequent Immune Response

In a natural infection, the host is invaded at the periphery and the virus gains access to host cells by breaking through the physical defences of skin and mucosal surfaces [106]. DCs, the specialized cells that capture and carry antigen from the site of infection to the LN whereupon they orchestrate the adaptive immune response, are found at a high frequency in the peripheral tissues, where they are influenced by the action of virus infection on the local environment [106, 108]. Viruses are obligate parasites; to survive they must enter (infect) the cells of the host where they then utilize the host cell machinery to produce the viral structural and non-structural proteins, RNA and DNA (DNA virus) they require to replicate [145]. To prevent such a hostile takeover, the mammalian system has devised ways to protect itself from infection, but at the same time, viruses and in particular, herpesviruses such as HSV-1, have evolved ways to get around these protective systems [5] including infection and impairment of the DC itself [146].

Infection of iDCs is mediated by interaction of DC-SIGN (DC-specific C-type lectin) with gB and gC of the virus. DC-SIGN also captures HSV-1 for transmission to permissive cells [147]. Earlier studies showed that only iDCs could support production of infectious HSV-1 viral particles, whereas in infected mature DC (mDC) only IE and E transcripts were generated. Additionally, maturation of DCs is interfered with by HSV-1 infection as was observed by complete ICP0-dependent proteosome-mediated degradation of the maturation marker CD83 [148] and an inhibitory effect on the induction of antiviral T cell responses and DC-mediated T cell proliferation [146]. To control the immediate infection and limit replication of the invading virus, the non-specific innate immune system of the host recognizes pathogen associated molecular patterns (PAMPs) of the virus [149]. The infected cells and innate-system cells such

as MΦs, pre-DCs and iDCs continually sample their surroundings to detect the presence of invading pathogens through pattern-recognition receptors (PRRs).

Recognition of PAMPs by PRRs of the infected cells initiates a cascade of signalling that results in the production of Type I inflammatory cytokines IFN-α and IFN-β to help the cells defend against infection or to limit viral replication even if this means death (apoptosis) of infected host cells. In addition to acting for the benefit of the infected cells themselves, these inflammatory cytokines are also released into the local environment and alert the surrounding cells to the presence of infection [150]. Secreted type I IFNs, IFN-α/β, activate the same antiviral mechanisms in the neighbouring cells, helping them to resist virus infection. As these responses are amplified by cells in the area, a local inflammatory antiviral state is established [151, 152]. HSV-1 infection can interfere with the induction of an antiviral state by reducing IFN-α secreted by DCs. This was observed in a mouse model where HSV-1 infection resulted in reduced ability for IFN-α secretion by otherwise high secreting plasmacytoid DCs [153].

Additionally, although expression of maturation/activation molecules of all the CD11c+ DC subsets was unaffected by *in vivo* infection of mice with HSV-1, the ability of these DCs to induce allo-T cell proliferation, and to secrete the Th1-type cytokine IFN-γ, particularly with respect to CD8+ cell secretion, was reduced yet Th2-type cytokine secretion was unaffected. Thus, through its action on the DC, HSV-1 infection induced a Th2-type response as opposed to the more desirable antiviral Th1-type response [153]. HSV-1 infection also blocked IFN-γ signalling in mDCs by impairment of the interferon-γ-induced, signal transducer and activator of transcription 1 (STAT1) signalling pathway. This was executed by down-regulation of the IFN-γ

receptor alpha chain (IFNGR1) mRNA (thus reducing expression of IFNGR1 on the cell surface), inhibition of STAT1 phosphorylation in response to IFN-γ, and the subsequent inhibition of downstream activation of the IFN-γ inducible interferon regulatory factor 1 (IRF-1) [154]; a molecule that as a transcription factor activates expression of IFN-β and that also functions as a transcriptional activator or repressor of genes involved in IFN-γ-mediated host response and immunity including MHC I antigen processing [154], regulation of apoptosis, tumor suppression, and DNA damage.

HSV-1 also could hamper DC induction of the antiviral response by interfering with migration of antigen-loaded DC from infected tissue to the secondary lymphoid organs. CCR7 and CXCR4 are receptors that are usually expressed by mDCs and that induce trafficking to their ligands CCL19 and CCL12 in the LN. Infection of mDCs with HSV-1, or of iDCs prior to maturation, caused either down-regulation of CCR7 and CXCR4 from the mDCs or failure of iDCs to up-regulate CCR7 and CXCR4 following maturation [155]. Chemokines are also secreted in response to virus and these provide the signals that recruit circulating MΦs, PMNs, natural killer cells (NK cells) and γδT cells to the site of infection [137]. Stimulation of these cells by the combination of IFN-α/β and PRR signalling promote sustained release of pro-inflammatory cytokines IL-1, TNF-α, and IL-6 [108]. NK cells recognize and kill virus-infected cells and release IFN-γ [156]. Chemokines such as CCL3 (MIP-1α), CCL4 (MIP-1β), CCL20 (MIP-3α) and CXCL10 (IP-10) are ligands for receptors (CCR1, CCR5, and CCR6) that are highly expressed by iDCs. Thus release of these chemokines serves to attract iDCs to the very site where viral proteins are being released from apoptotic infected cells [106]. Within this “stew” of inflammatory molecules and signals, iDCs are taking up antigenic viral proteins, processing them

and being influenced as to the type of adaptive response they will ultimately induce [106]. Thus any interference by the virus on the DCs' ability to secrete or be stimulated by cytokines ultimately interferes with the effectiveness of the immediate antiviral response and the subsequent adaptive response. Fortunately, although this initial delay in the generation of immune responses to HSV at peripheral sites is accomplished by viral disruption of DC function, it is ultimately overcome by bystander DC maturation (soluble factors secreted by DCs after infection induced DC maturation of the surrounding uninfected DCs) [157] and the ability of non-infected and specialized subsets (ie. CD8α) of DCs to cross-present exogenous viral antigen on MHCI molecules--a method by which CD8 T cell responses can be induced/achieved, viral immune evasion mechanisms notwithstanding [157, 158].

1.2.5 Modulation of Immune Response by DCs

Influenced by the local inflammatory antiviral state, iDCs undergo maturation and secrete successive waves of cytokines that recruit Mo, lymphocytes and more NK cells to the area [159]. Viral infection typically results in sufficient inflammation that strong recruitment of lymphocytes and NKs occurs, and these infiltrating cells then begin secreting large amounts of IFN-γ. In this environment of IFN-γ, maturing DCs develop; travel to the LN and up-regulate the co-stimulatory molecules that enable them to prime an adaptive immune response. For most viral infections including herpesviruses, this involves priming naive T cells and driving $CD4^+$ cells toward an antiviral Th type I (Th1) phenotype [106]. In the absence of these signals, anergy or tolerance is induced (not desired for an adaptive antiviral response) [160]. Induction of CD8+ CTLs requires Th1 assistance, so induction of sufficient Th1 cells is critically important for an anti-viral CD8+ response [161]. Mature DCs (described above) provide the signals that activate

naïve B cells to plasma cells (IL-12, IL-6/soluble IL-6Rα), and CD40-activated memory B cells (IL-6/soluble IL-6Rα) to IgG-secreting cells [162, 163]. When antigen-specific CD4 T cells and antigen-specific B cells interact with mature DCs through BAFF/Blys-L (on DC and T-cells); B cells proliferate and antibody is produced and secreted [164, 165]. Mature DCs readily prime Th cells. Thus Th activated by mature DCs complete the immune response by interacting with B cells (antibody production), MΦs (activate them), and infected cells (lysis) [137].

1.3 Bovine Beta-Defensins

1.3.1 Host Defense Peptides

Host defense peptides (HDPs) are small mostly cationic molecules that participate in the early host defence response against microorganisms. Since the primary function of these peptides was originally considered to be their antimicrobial activity, they are also known as antimicrobial peptides (AMPs). HDPs are molecules of innate immunity, so called because they are gene-encoded, quickly expressed or delivered anti-infection tools of the host. AMPs of plants (thionins), that were toxic when injected in animals, were isolated in the 1970s and since then non-toxic plant AMPs called plant defensins were discovered and characterized [166, 167]. Similar peptides have been described in insects [168], arthropods and molluscs [169], fish and amphibians [170] and vertebrate animals [170, 171]. More than 2000 of these natural peptides have been found in eukaryotes and bacteria [172], and over 700 AMPs have been isolated from mammalian tissues [173]. HDPs possess direct antimicrobial as well as anti-infective, anti-tumor or immunomodulatory properties [172, 174] and are divided into two main groups: (1)

cathelicidins and (2) the α- defensins and β-defensins [175]. The diversity of functions fulfilled by some of the mammalian α- and β-defensins in host defense are illustrated in Figure 1.4.

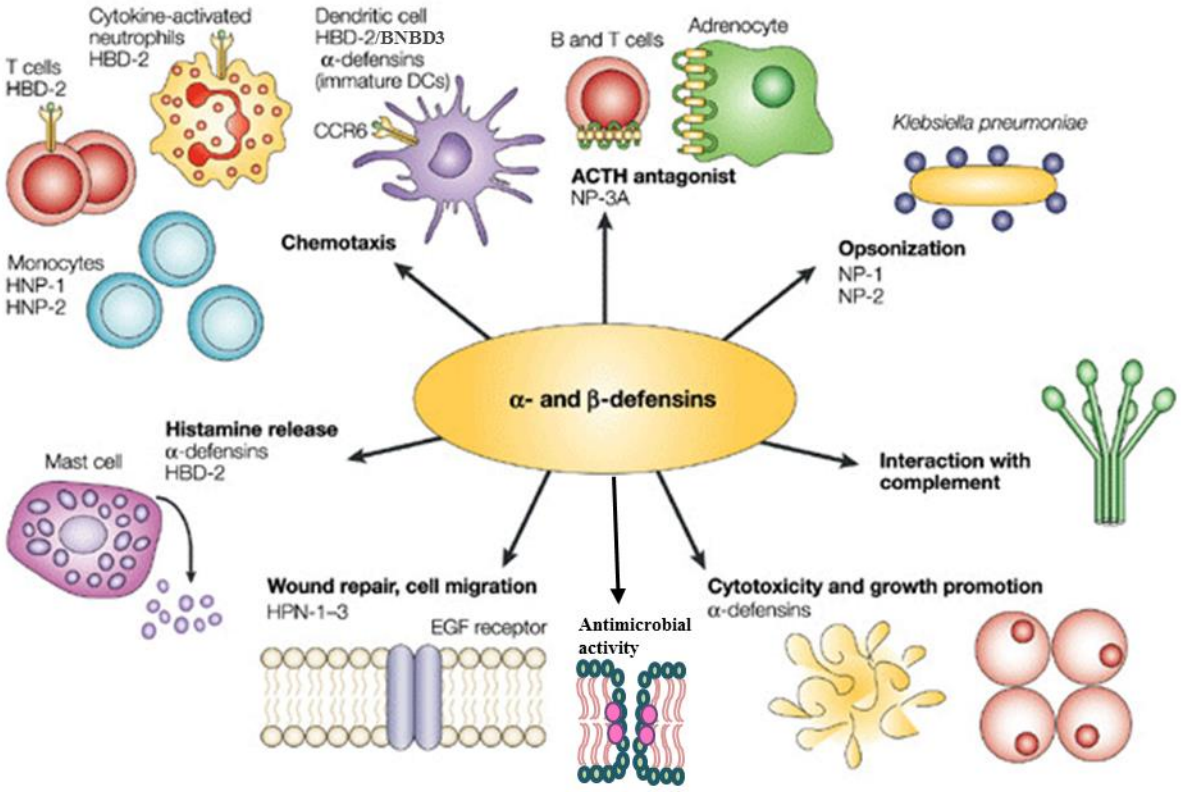

Figure 1.4 Functions of defensins. In addition to antimicrobial activity, defensins participate in host defence by carrying out a variety of functions that contribute to the innate and adaptive immune response. (Adapted by permission from Macmillan Publishers Ltd.: Nature Reviews Microbiology, Lehrer (2004) [176]. http://www.nature.com/nrmicro/index.html)

1.3.1.1 Cathelicidins

Cathelicidins are a diverse family of small (12-100 aa) cationic amphipathic peptides that derive their name due to the homology of their evolutionarily conserved pro-sequence to the porcine neutrophil protein cathelin. They are produced as precursor, with an N-terminal signal peptide, a

highly conserved cathelin domain, and a variable (sequence and structure) C-terminus that is cleaved off to give the mature peptide [177, 178]. Once the signal peptide has targeted the cathelicidin to storage granules or to the exterior of the cell it is cleaved off. Further processing to form two disulfide bridges yields a storage form holoprotein or "pro-protein". When the cell is activated, proteolytic cleavage of the holoprotein at a cleavage site between the cathelin domain and C-terminal peptide occurs; and the active mature peptide is released [179, 180]. The four possible structures of mature C-terminal cathelicidin peptides and an example of each are: α-helical (human LL-337), proline-rich (bovine Bac-5), disulfide bonds and/or β-sheets (bovine Protegrin) and tryptophan-rich (bovine Indolicidin) [177, 180].

1.3.1.2 Alpha-Defensins

Mammalian defensins are a family of secreted cationic and highly disulfide-bonded peptides. They are divided into three subfamilies α-, β-, and Θ defensins based on the linear location and spacing of their cysteine residues and disulfide pairing of their six conserved residues [175]. The disulphide-paired cysteines of the α-defensins are linked Cys [1-6], Cys [2-4], and Cys [3-5]; and for the β-defensins they are linked Cys [1-5], Cys [2-4] and Cys [3-6]. The Θ-defensins are structurally dissimilar and derived from mutated α-defensin genes. As such they are backbone cyclic octadecapeptides that are stabilized by three disulfide bonds [181]. In Table 1.2 some of the known mammalian α- and β-defensins are listed with their characteristics described [182-185]. The first α-defensins were discovered in rabbit aMΦ and they had microbicidal activity and antiviral activity toward HSV [186, 187]. The same peptides could not be found in rabbit peritoneal MΦs [188], but were isolated from rabbit neutrophils and so were renamed NP-1 and NP-2. With the additional four peptides that were discovered in rabbit peritoneal neutrophils, a

total of six α-defensins (NP1-6) were isolated and characterized from rabbit [189]. Currently there are six known human α-defensins (HNP1-6). HNPs1-4 derive from the cytoplasmic granules of polymorphonuclear leukocytes (PMN; neutrophils), and have HSV-1 antiviral and broad spectrum antimicrobial activities [190]. HDP5 and 6 are expressed in the small intestinal Paneth cells (specialized secretory cells at the base of the small intestinal crypts) [191], as well as in epithelial cells of the female genital tract and are known mediators of antimicrobial defense [192]. Mouse α-defensins (cryptidins) are also expressed by small intestinal Paneth cells [193, 194] but not by PMNs [195]. Mouse cryptidins 1-6 have potent antimicrobial activity against specific microorganisms that is initiated by the microbially stimulated release of their cryptidin-containing granules from the Paneth cells to the lumen of the crypts of Lieberkhun in the small intestine, where the α-defensins also influence mucosal immunity [193, 196, 197]. Interestingly, neither α-defensins nor the genes encoding for them have been found in cattle [198].

Table 1.2 Mammalian α- and β-defensins: sources, expression, and immune functions

							Activity relevant to immunity	
Defensins	**Type**	**Host**	**Cell/Tissue Source**	**Synthesis**	**Release**	**Regulatory Elements**	**In vitro**	**In vivo**
HNP1-3	α	Human	N, CD8 T	C I	D S	C/EBPa	*Microbicidal *Antiviral *Chemotactic *Promote phagocytosis *Mast cell degranulation *Stimulate cytokine, chemokines *Regulate complement activation *Inhibit ACTH receptor and glucocorticoid production	*Recruiting immune cells *Inducing cytokines *Enhancing Ag-specific humoral and cellular IRs *Resistence to bacterial invasion
HNP4	α		" "	"	"	C/EBPa		
HD5	α		Paneth cells/GI, GU tract	C	D	NF IL-6		
HD6	α		" "	C	D	NF IL-6		
Cryptdins	α	Mouse Rat	Paneth cells/GI tract	C	D	ND[a]		
NP1-2	α	Rabbit	aMΦ	C	D	ND		
HBD1	β	Human	K, EC/GI, GU,OM,P, Ki,Co,L,T, Sk, Ear, Si	C I	S	ND	*Microbicidal *Chemotactic via CCR6 (HBD2, mBD2) *Mast cell degranulation *Stimulate cytokine, prostaglandin *DC maturation via TLR4 (mBD2)	*Enhancing Ag-specific humoral and cellular IRs *Resistence to bacterial invasion
HBD2-4	β	Human	K, EP/,L,T, Sk, Eye, Si	I	S	NF-κB		
mBD1	β	Mouse	Gut,H,Ki,L,aMΦ,U	C	-	ND		
mBD2	β	Mouse	K,U,H,A	I	-	ND		
mBD3	β	Mouse	Si,L,Li	I	-	ND		
pBD1	β	Pig	A,GI,OM,To	C	-	ND	-	-
sBD1	β	Sheep	GI,T	ND	-	ND	-	-
sBD2	β	Sheep	GI	ND	-	ND	-	
TAP	β	Cow	EC/T, aMΦ	I	S	NF-Kb, NF IL-6	*Microbicidal	-
LAP	β	Cow	To,T	I	S	NF-Kb, NF IL-6		-
EBD	β	Cow	Si	I	S	NF IL-6		-
BNBD1-3, 6-11	β	Cow	N	C	D	-		-
BNBD4	β	Cow	N, aMΦ, T, dSi, L Sp	C	D	-		-
BNBD5	β	Cow	N, aMΦ	C	D	-		-
BNBD12,13	β	Cow	N,T,C,dSi	C	D	-		-

N= neutrophil, C= constitutive, I= inducible, D= degranulation, S= secretion, C/EBPA= CCAAT/Enhancer-binding protein, GI= gastrointestinal, GU= genitourinary, NF= nuclear factor, K= keratinocyte, EC= epithelial cell, OM= oral mucosa, P= pancreas, Ki= kidney, Co= colon, L= lung, T= trachea, Sk= skin, Si= small intestine, dSI= distal Si, H= heart, U= uterus/female reproductive organs, A= airway, Li= liver, To= tongue, Sp= spleen, [a] Not determined, - Not known

(Adaped from Diamond and Bevins (1998) [185], Kaiser and Diamond (2000) [182], Ganz (2003) [184], Yang (2004) [183]).

1.3.2 Beta-Defensins

β-defensins are cationic, membrane active, antimicrobial proteins of the innate immune system that participate in defense against microbiological pathogens [199]. They are 38 to 42 amino acids in length (cattle), with three intra-molecular disulphide bridges [199-201]. As mentioned above, they differ from the α-defensins by their disulfide pairing and they are linked Cys [1-5], Cys [2-4] and Cys [3-6]. Also, where biosynthesis of the α-defensins involves cleavage of a signal peptide from the prepro-α-defensin to produce a pro-α-defensin, and this pro-peptide has little or no antimicrobial activity *in vitro* due to the nature of the pro-piece that is large (about 40 aa) and anionic [202]; the β-defensins have a much shorter pro-piece separating the signal and mature peptide. This would result in a pro-β-defensin with a more cationic nature and would be expected to result in a different way of trafficking within the cell as compared to a pro-α-defensin [175]. β-defensins are evolutionarily older than the α-defensins and it is thought that the β-and the α-defensins evolved from a common β-defensin ancestor [203]. From the common ancestor it is postulated that α-defensins came about by repeated gene duplication of β-defensin coupled with positive diversifying selection which is the evolutionary response to the changing makeup of microbes in the host habitat [204, 205]. Perhaps the above explain why β-defensins are found in a much broader range of species [188] and expressed in a wider variety of tissues than are α-defensins [175, 182, 184].

1.3.2.1 Human Beta-Defensin

Although a large number of new human β-defensin genes have been reported [206], there are currently six known β-defensins and the first four, namely human β-defensin 1 (hBD1), human β-defensin 2 (hBD2), human β-defensin 3 (hBD3), and human β-defensin 4 (hBD4) have been characterized in detail. hBD1, hBD2 and hBD3 are mainly expressed in the skin and in the epithelial lining of the urinary and respiratory tracts although hBD3 is also expressed in non-epithelial cells of the heart, liver and placenta [207, 208]. hBD4 and the more recently discovered human β-defensin 5 (hBD5) and human β-defensin 6 (hBD6) [209, 210] are primarily expressed in the testis and epididymis [208]. All human β-defensins characterized have the ability to kill or inhibit *in vitro* bacteria and fungi although much of this ability is salt- and plasma protein-sensitive. Additionally antiviral effects have been reported, particularly with respect to *in vitro* inhibition of human immunodeficiency virus (HIV) -1 by hBD2 and hBD3 [211, 212]. HIV-1 induced expression of hBD2 and hBD3 in human oral epithelial cells [212] and hBD3 was able to induce maturation and Th1 skewing in human langerhans cell-like DCs (LC-DC) [213]. Thus it is tantalizing to think that a β-defensin might have been part of the soluble factors secreted from DCs after HSV-1 infection that had the ability to induce maturation and increase IL-12 secretion of uninfected iDCs [157]. In addition to effects on maturation, human β-defensins can also contribute to the induction of acquired immune responses by recruiting immune cells to sites of inflammation. Human β-defensin 2 was chemotactic for iDCs and memory T cells through its interaction with CCR6 expressed by the cells [214] and hBD3 was chemotactic for CCR2-expressing cells such as Mo, MΦs and neutrophils [215].

1.3.2.2 Murine Beta-Defensin

Many β-defensins have also been described in the mouse [185], and this been particularly useful to study and predict the effects of β-defensins in human and other species. Murine β-defensin 4 (mBD4) and Murine β-defensin 14 (mBD14), the reported orthologs for hBD2 and hBD3 respectively, interact with CCR2 and induce chemotaxis of human Mo and mouse peritoneal exudates cells [215]. Much work was done to establish the immunomodulatory effects of murine β-defensin 2 (mBD2) at the time when it was thought to be the homolog for hBD2. In a toll-like receptor (TLR) 4-dependent manner, mBD2 increased activation and maturation of mouse iDCs as evidenced by upregulation of costimulatory molecules and CCR7 with an increased Th1-polarizing proinflammatory cytokine profile [216]. mBD3 was chemotactic for murine bone marrow-derived DCs, and induced protective anti-tumor immunity in mice immunized intradermally with an mBD2/ tumor antigen fusion construct [217]. In a murine model, intradermal (ID) DNA immunization with an mBD2/gp120 fusion construct induced antiviral responses including mucosal CTL and neutralizing antibody to the HIV-1 envelope glycoprotein gp120 [218].

Immunomodulatory characteristics such as the ability to chemo-attract iDC and to activate and mature iDC to mDC have not been established for the majority of β-defensins of large animals (ie cattle). If it was shown that β-defensin could attract iDCs of large mammals and subsequently mature them to boost immune responses, then β-defensin would have potential as an adjuvant in vaccines for cattle and other large animals.

1.3.2.3 Bovine Beta-Defensin

In cattle, sixteen β-defensins have been discovered, thirteen are produced by bovine neutrophils (BNBD 1-13) [219]; one is produced by tracheal epithelial cells (TAP) [199]; another is produced by squamous epithelial cells of the tongue (LAP) [200]; and one is produced by the epithelial cells of the intestine (EBD) [201]. As with all other mammalian β-defensins, the bovine β-defensins are cationic and antimicrobial, but the mechanism has not yet been elucidated. Additionally, the β-defensin genes have two exons. The first exon encodes the 5'UTR and leader domain of the pre-pro-peptide. The second exon encodes the mature peptide [220, 221]. Expression of bovine β-defensin is inducible by endotoxin, LPS and TNF-α [222-224]. An overview of α- and β-defensin translation and post-translational processing is shown in Figure 1.5. Similar to the cathelicidins, the translation product is an inactive precursor (pre-pro-peptide) with an N-terminal signal sequence, a short pro-piece and a C-terminal mature peptide that is released as an active peptide when it has been cleaved by proteolytic processing [225]. BNBDs 1-13 are constitutively expressed (produced and stored) by the neutrophil during the promyelocyte stage, and are released in response to inflammatory mediators (LPS etc.) [185]. The epithelial β-defensins TAP, LAP and EBD, are inducibly expressed under conditions of infection and inflammation [200, 201, 226].

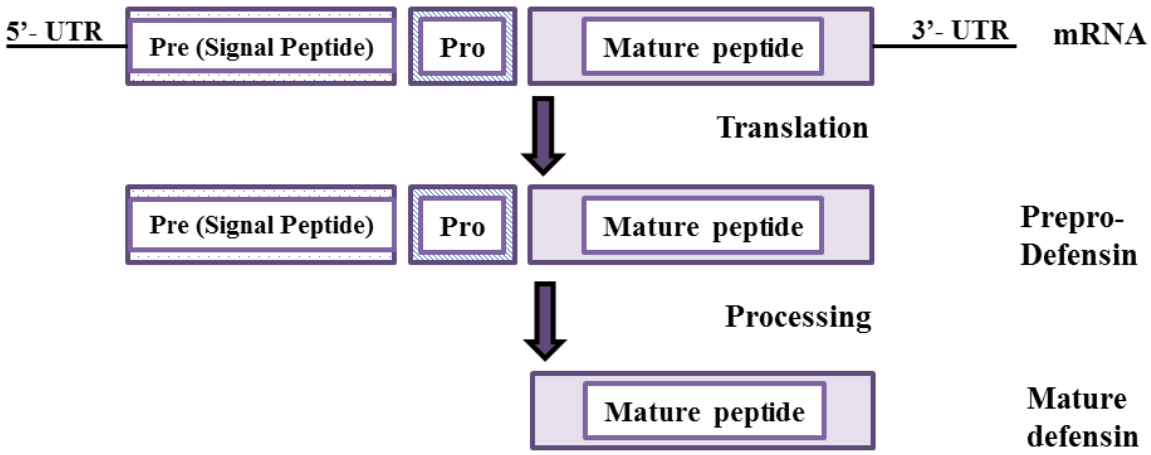

Figure 1.5 A schematic of translation and post-translational processing of defensins. Defensins are translated as larger precursor polypeptides consisting of the prepiece, the propiece (often anionic), and the mature peptide. The prepiece is a highly hydrophobic signal peptide that is proteolytically cleaved in the Golgi body. The prepro region is proteolytically removed as a post-translational modification (hBDs, TAP, LAP, EBD) . Cleavage of the propiece differs for different defensins. For HNPs (and BNBDs) the propiece is proteolytically removed in the Golgi body, and the mature peptide is sorted to the primary granules of neutrophils for storage. HNP5 is stored in granules of Paneth cells and is released as the proform. Interestingly, removal of the propiece of HD5 and cryptidins occurs extracellularly and is mediated by trypsin and matrilysin respectively. The enzyme(s) responsible for the removal of the propiece of β-defensin is unknown. Both α- and β-defensin peptides can be further processed by removal of individual amino acids from the N-terminus of the mature peptide. This variable processing can generate multiple forms of the mature peptide, and likely contributes to biological diversity. (Adapted from Yang et al. (2004) [183]).

Based on gene structure characteristics such as site of expression, elements of regulation, intron size, and sequence similarity, bovine β-defensins are grouped with the epithelially-expressed human β-defensin 2 (hBD-2) [185]. Recently, bovine β-defensin genes were screened for molecular evolution. EBD and TAP were unchanged, and thus are considered subject to stabilizing negative selection (removal of changes/alleles that would be detrimental), while BNBD5 and BNBD9 were evolving under positive selection. Even with variances, or under positive selection pressure, all the peptides conserved the β-defensin consensus characteristics:

the triple-stranded β-sheet, the general fold, and the short amino terminal α-helix [227]. Interestingly, a novel β-defensin-like gene called bBD1 gene was found using primers designed to clone cDNA of an AMP expressed in bovine mammary epithelial cells that would be similar to hBD1. The cDNA predicted a 69 amino acid pro-peptide similar to hBD1. Gene organization study showed that the novel bBD1-gene had one long intron (8547 base pairs (bp)) as does the human β-defensin 1 (hBD1) gene (6962 bp) whereas the other bovine β-defensins had intron lengths of approximately 1500 bp. There was 57% similarity in sequence between bBD1 and hBD1, but only 34% when compared to LAP; whereas LAP showed 78, 86, and 89% similarity to BNBD4, EBD and TAP, respectively. The bBD1 transcript was detected in the teat, mucosa, kidney, vagina, ovary, oviduct and colon and a synthetic bBD1 peptide had antibacterial activity against *E. coli.* [228]. The results of this study suggest a homology in structure and function of β-defensins between species and additionally suggest that more bovine β-defensins might exist, although isolation of the mature peptide from tissue still should be required as proof-positive.

1.3.2.3.1 Bovine Beta-Defensin 3

BNBD3 is one of thirteen bovine neutrophilic β-defensins that were numbered according to their increasing retention times on RP-HPLC. Based on the original isolation and characterization of these peptides from bovine neutrophils, BNBD3 is a mature 42-residue peptide with an N-terminal pyroglutamyl residue and a calculated molecular weight of 4809 (UniprotKB/Swiss-Prot accession number P46161). The peptide conforms to the β-defensin consensus that consists of 27 residue positions including the 11 absolutely conserved residues, the 16 residues where substitutions are conservative or limited, and the 3 disulphide bonds specified Cys [1-5], Cys [2-4] and Cys [3-6]. BNBD3 is the most abundant of the 13 neutrophilic β-defensins, present at

approximately 2.2 mg / 4.9 mg (equals 10^{10}) neutrophils or 45%. It has antimicrobial activity against *E. coli* ML35 and *S. aureus* 502A [219]. It is a basic peptide with a predicted pI of 11.71 and a net charge of 10^{+}.

1.3.3 Beta-Defensins In Immune Modulation

Protective immunity to pathogens depends on accurate yet flexible immune responses tailored to the type of pathogen and the infected tissue. One of the very first steps to accomplishing immunity is to get the required immune cells to where they are needed. Defensins are potently chemotactic for a variety of immune cells, perhaps due their structural similarity to cytokines [229]. Specifically, hBD2 can recruit iDCs and the memory subset of peripheral blood (CD4+/CD45RO+) T cells [214]. hBD2-3 can recruit neutrophils and mast cells and induce mast cell degranulation [230-233]. Few receptors for immune cell recruitment by β-defensins are known with the exception of CCR6 for iDC and (CD4+/CD45RO+) T cells by hBD2 [214], and CCR2 for Mo by hBD3 [215]. Glycosaminoglycans may serve as a type of receptor to enhance chemotaxis of cells to β-defensins [234]. Production and release of β-defensin by cells in response to infection or trauma encourages migration of immature dendritic cells (iDC) from the circulation to the tissue [217, 218]. As more iDCs migrate into the area, it becomes more likely that a pathogen will be taken up by iDCs. Also, β-defensin peptides may form complexes with proteins, peptides and dying cells from infection and effectively target them to the iDC resulting in improved trafficking through cellular compartments, processing and presentation that includes cross presentation on MHC I molecules [161].

β-defensin also induces cells to express pro-inflammatory cytokines and chemokines [217, 235]. Treatment of keratinocytes with hBD3 induced CCL2 (MCP-1), CCL20 (MIP-3α), and CXCL10 (IP10) [236]. Treatment of Mo with hBD3 induced IL-8, IL-6, and IL-1β through Toll like receptors (TLRs) 1 and 2 [237]. The murine β-defensin mBD2 acts directly on iDCs as an endogenous ligand for TLR 4 causing up-regulation of costimulatory molecules and maturation of DCs [216]. LL37 and several other cell-penetrating cationic peptides were recently found to enhance signalling by TLR3 and enable TLR3 to respond to dsRNA stimulation [238]. TLR3 was expressed by the CD8α DCs that were involved in priming HSV-1 CD8 T cells; and in that study, for priming to occur, there had to be co-expression of TLR3 and the MHC class I-restriction element by the presenting CD8α DC [239]. It is intriguing to think that β-defensins might also be acting on TLR3 of CD8α DCs and that this might explain at least in part, the stimulatory ability and Th1-polarized responses of DC treated with the cationic defensin peptide mBD2 [216], and the potent *in vivo* MHCI-restricted primary T cell responses primed in mice by vaccines encoding mBD2 [161].

The influence of these substances on the iDC induces maturation, and the DCs become licenced to migrate from the tissue to the LN where they present antigen to prime naïve T-cells [137]. Sufficient signals are critical to maturation. Without these, anergy and/or tolerance result [240]. Mature antigen presenting DCs are potent, and uniquely capable of initiating both humoral and cell mediated primary immune responses through cross presentation of antigen on both MHC I and MHC II molecules [110, 114, 217]. β-defensin has potential to provide both the chemotactic impetus and the maturation signal and thus satisfy the above requirements in a DC targeting immunization strategy for BoHV-1 that mimics natural infection.

1.4 DNA Vaccines

A DNA vaccine is described as an antigen encoding plasmid that, when introduced into the body, results in *in vivo* expression of the antigen, with a subsequent antigen-specific immune response [241]. DNA vaccines consist of a recombinant foreign gene cloned into a double-stranded, closed-circular bacterial plasmid vector. The plasmid generally contains an origin of replication (for amplification in bacteria), a bacterial antibiotic resistance gene (to allow for selection), the gene(s) of interest under the transcriptional control of a (viral) promoter and enhancer sequences (to obtain strong expression in mammalian cells) followed by a mRNA polyadenylation sequence (to stabilize the mRNA transcripts) [242].

1.4.1 Benefits of DNA Vaccines

Compared to other types of vaccines, DNA vaccines are cost-effective; they can be designed, manufactured and stored with relative ease [241]. They are non-infectious and do not promote inflammation at the site of immunization; important factors in vaccinations for food animals [243]. DNA vaccines can be used as marker vaccines to differentiate vaccinated and infection-exposed animals in eradication programs [243]. They also have potential to initiate immunity in neonates born to immune mothers [244]. DNA vaccines stimulate both humoral and cellular immunity, and thus promote development of a balanced immune response [108, 243]. In the induction of humoral and cellular immune responses, DNA vaccines seem to mimic the effects of natural infection (or live attenuated vaccines) in at least two ways: they are able to induce MHC class I-restricted CD8 T-cell (CTL) responses [241, 245]; and, they have the ability to

generate neutralizing antibodies to epitopes formed by non-contiguous regions which suggests that antigen expressed *in vivo* after DNA vaccination can assume a native configuration [246].

1.4.2 Immune Responses Induced by DNA Vaccines

In 1990, Wolff et al showed that injection of pure plasmid or "naked" DNA into the muscles of mice resulted in long-term expression of encoded reporter genes [247]. Induction of antigen-specific immune responses with plasmid DNA was first reported by Tang et al, when mice immunized with plasmid encoding human growth hormone (hGH) developed an immune response specific for the hGH [248]. Since then, there have been more than 600 reports of successful induction of immune responses in animals given DNA vaccines [249]. Although many of these studies used the mouse as an experimental model, and results with large animals have not been as encouraging [243], DNA immunization has shown efficacy in primates and veterinary species [250], and human clinical evaluations have been initiated for HIV, hepatitis B virus (HBV) and influenza viruses [108]. The successes in induction of appropriate and protective immune responses in mice as a result of DNA vaccination, have not been equalled when the technology has been transferred to large animals [249]. It has been suggested that a more robust response could be obtained by targeting the DNA vaccine or its expressed antigen to professional APCs such as DCs [242, 251].

1.4.2.1 Antibody Responses

DNA vaccines tend to induce less potent antibody responses in many different animal models than they do cellular responses [252]. This may be due to the encoded antigen getting expressed from within the transfected cell; thus as an "in-cell" antigen, it may be more likely to be

processed via the endogenous pathway with the subsequent peptides loaded onto and presented by MHC I molecules. It is only when antigen is released outside the cell, or when taken up from a dying cell, that it becomes available as exogenous antigen to the MHC II pathway (induction of required Th cells) or to the B cell receptor. Perhaps humoral responses are limited by this consequent limited availability of free antigen. This would seem sensible as secreted antigen is more likely to become "free" antigen, and humoral responses to DNA-encoded secreted antigens are higher than to their cell-associated counterparts [44, 253, 254]. Yet it has also been reported that DNA vaccination induction of CTLs has led to enhanced humoral responses [255] which suggest a possible synergistic generation of both humoral and cellular arms of adaptive immune responses. Protective antibody responses have also been observed after immunization with a HBV DNA vaccine that also induced antigen-specific Th cells and CD8 T cells [256]. Peak antibody responses occur 4-12 weeks after DNA vaccination which is relatively slow, but the response tends to be of long duration [257], and the antibody tends to be of good neutralizing ability, and to have good avidity (reviewed in [241]). In comparative studies, humoral responses have been greater in response to immunization with a sub-lethal dose of live virus [258], or to a protein based vaccine [259], than to the antigen-equivalent DNA vaccine. Antibody subtypes of IgB, IgA and IgG are generated in response to DNA vaccination and the subclass is typically influenced by the Th1 cytokine bias of DNA vaccines such that in mice antibody of subclass IgG2a/b was more commonly observed than was IgG1 [260]. Secreted DNA-encoded antigen tends to induce antibody of the IgG1 subclass [253], as does vaccination via gene gun [260]. Intradermal delivery by gene gun could be modified to induce a balanced IgG1 and IgG2 response by providing additional immunostimulatory signals either by co-delivery of cytokine or by increasing immunostimulatory DNA [261].

Interestingly, how the DNA vaccine is given also influences the type of immune response and this may be related to the type and location of cell that is transfected and subsequently expresses the antigen. In mice, intramuscular (IM) injection induced mainly humoral responses to DNA-encoded antigen, that were not dependent on expression of antigen by the muscle tissue at the injection site; whereas humoral responses from gene gun delivery were lower and required expression of antigen by transfected cells in the skin. Thus skin cells at the site of immunization (gene-gun delivery), but not muscle cells at the site of immunization (IM delivery) were important for DNA-raised responses [262]. In large animals such as cattle and pigs, ID delivery has been shown to be more effective than IM (reviewed in [263]). In cattle naked/unadjuvanted DNA vaccines delivered ID to the ear improved antibody responses when compared to IM delivery [44]. Also, the effect of gene gun delivery of DNA vaccines on humoral responses of cattle is less clear. In one study, no improvements in humoral responses were attained when cattle were primed with a DNA vaccine delivered by gene gun to the genital mucosa (intravulvomucosally) [264]. When the effects of ID (to the skin of the hip) versus intravulvomucosal gene-gun delivery of a DNA vaccine were compared, humoral responses were low in both groups, although intravulvomucosal delivery was able to prime humoral responses [265]. However, in another study, humoral responses after gene gun delivery of a DNA vaccine to the skin were determined to be of sufficient magnitude, were long lasting and followed a general pattern of being primarily Th2 biased (predominantly IgG1 over IgG2) [263].

1.4.2.2 Cellular Responses

How cellular responses are induced by DNA vaccines can be likened to how they are induced by infection by virus or intracellular bacteria. In both cases, pathogen or DNA-encoded proteins are

produced within the host cell. Thus DNA vaccine-encoded proteins have a propensity for processing via the endocytic host cell pathway with subsequent loading to and presentation by MHC I molecules. There are however, three ways that antigen encoded by a DNA vaccine can be obtained and this has an effect on how it is processed and presented and thus an effect on the type of immune response elicited: (1) Direct priming by somatic cells that occurs when transfected resident tissue cells produce the DNA vaccine-encoded antigen; process the antigen through the endogenous pathway, and present antigen on MHCI molecules to specific CD8+ T cells. This type of "priming" does not prime naïve $CD8^+$ T cells since the somatic cells lack the co-stimulatory molecules required to initiate an immune response [266, 267], but continued expression of antigen by somatic cells most likely serves to augment or maintain the immune response after DNA vaccination [268]. (2) Direct transfection of professional APCs (bone-marrow derived cells such as DCs) that occurs when DCs at either the vaccination site or the (LN are transfected, produce and process the antigen through the endogenous pathway and present antigen on MHC I molecules. There is also evidence that the endogenous antigen can enter the exogenous pathway by cytosolic degradation or antigen regurgitation and thus be presented on MHC II [241, 269]. These DCs when mature express the co-stimulatory molecules CD80, CD86, CD40 and thus by presenting on MHC I they can prime naïve $CD8^+$ T cells including induction of CTLs [270, 271], or by presenting on MHCII they can prime naïve $CD4^+$ Th cells [269]. (3) Acquisition of exogenous antigen that occurs when DCs take up the DNA encoded antigen that has been secreted from transfected somatic cells (exogenous antigen) or when DCs phagocytose transfected dying cells. In this case antigen is normally processed through the exogenous pathway and presented on MHC II but DCs have a specialized ability to "cross-present" and by this method, the antigen can escape or is released from the endosome to the cytosol where it is

processed through the endogenous pathway and presented on MHC I [111, 272]. Additionally, antigen-loaded MHC I molecules obtained from the dying cells can be recycled, allowing for peptide exchange in the MHC II compartments (MIICs) [273] or can be "cross-dressed" [274] and expressed on the surface of the DC. Thus, acquisition of exogenous antigen by DCs can result in priming of both naïve $CD4^+$ Th cells and naïve $CD8^+$ T cells including CTLs dependent on whether the antigen is presented on MHC II or MHC I molecules respectively. In the case of cross-dressed antigen, this mechanism does not prime naïve cells, but rather activates and thus boosts the response of memory CD8+ T cells [274].

1.4.3 DC-Mediated DNA Vaccines

Live attenuated viral vaccines, and vaccines consisting of a related but less virulent strain, can mimic natural infection, and effectively induce a protective, balanced immune response [242]. In the induction of humoral and cellular immune responses, DNA vaccines also seem to mimic the effects of natural infection [241, 245]. Despite these benefits, DNA vaccination typically requires large amounts of DNA. Additionally, in outbred, large animal species DNA vaccination has not always been successful [243]. Since very small amounts of antigen are produced as a result of DNA vaccination, and transfection is an inefficient process, efforts to increase the likelihood and the magnitude of an appropriate immune response are rational. It is possible that a robust response could be obtained by engineering the DNA vaccine so that its' expressed antigen will attract iDCs. The transfected and antigen-loaded DCs would then act as adjuvants to generate an immune response and consequent resistance to infection [106, 275].

1.4.4 Vaccination Strategies for BoHV1

Currently available MLV and KV BoHV-1 vaccines have been shown to reduce clinical symptoms but do not afford adequate protection from infection [98, 99, 276]. Of the two types, the MLVs are considered more effective, as they stimulate not only humoral immunity, as is the case with KVs, but also CMI [277, 278]. Disadvantages of the current MLVs include: 1) a requirement for proper storage and handling (cold chain), 2) they can cause abortions and therefore cannot be used for all cattle, 3) vaccinated animals may shed infectious virus and 4) there is a risk of development of latency [242, 279] .

1.4.5 Use of Defensin In a BoHV-1 DNA Vaccine to Modulate Immune Response

The events that lead to the induction of immune responses after inoculation with a DNA vaccine are not fully understood [241, 242], but there is increasing evidence that DCs act as adjuvants to initiate immune responses to DNA immunization [108, 241, 269, 275, 280]. Steinman et al., 2006, identified three "intricate and innate" properties of DCs that account for their roles as sentinels and sensors in the immune system: 1) special mechanisms for antigen capture and processing, 2) the capacity to migrate to defined sites in lymphoid organs to initiate immunity, and 3) their rapid differentiation or maturation in response to a variety of stimuli [281]. These are the very properties that make them desirable vaccine targets.

Primary antigen-specific, CMI responses are characterized by activation and expansion of T cells to Th and CTLs, and only occur when antigen is presented to T cells by professional APCs such as DCs in the context of additional immunostimulatory signals [241, 242, 282]. Because the quantity of antigen produced *in vivo* after DNA vaccination is very small (picogram to nanogram

range), the APC responsible for initiating an immune response must display great efficiency in antigen capture, processing and presentation [241, 283]. As these are characteristics that are inherent in DCs, and given the iDC chemotactic nature of human and mouse β-defensins, it follows that bovine β-defensin, if chemotactic for bovine iDC, might have potential to increase iDC-uptake of the encoded antigen of a DNA vaccine in cattle. By providing the required maturation signal, the β-defensin would effect maturation to result in DC priming of naïve T cells. It makes sense then, to explore this possibility by designing a DNA vaccine with β-defensin in such a way that iDCs will be attracted, and that the vaccine and/or its expressed antigen will effectively be "targeted" to the iDC and will provide co-stimulatory signals for DC maturation.

At each step, the expected impact/benefit of the β-defensin on DC function and subsequent immune response to DNA vaccination is depicted in Figure 1.6, and could be described as follows. (1) After vaccination, plasmid would be internalized by resident skin cells and the encoded antigen synthesized in transfected local cells would be released or obtained by phagocytosis of dying skin cells [108, 241]. At this step, if an iDC chemotactic bovine β-defensin was complexed to the DNA vaccine, we would expect infiltration of greater numbers of iDC and perhaps pre-DC with subsequent increased uptake of the plasmid by these cells. If encoded by the DNA vaccine, we would expect iDC to be attracted to the site by expressed

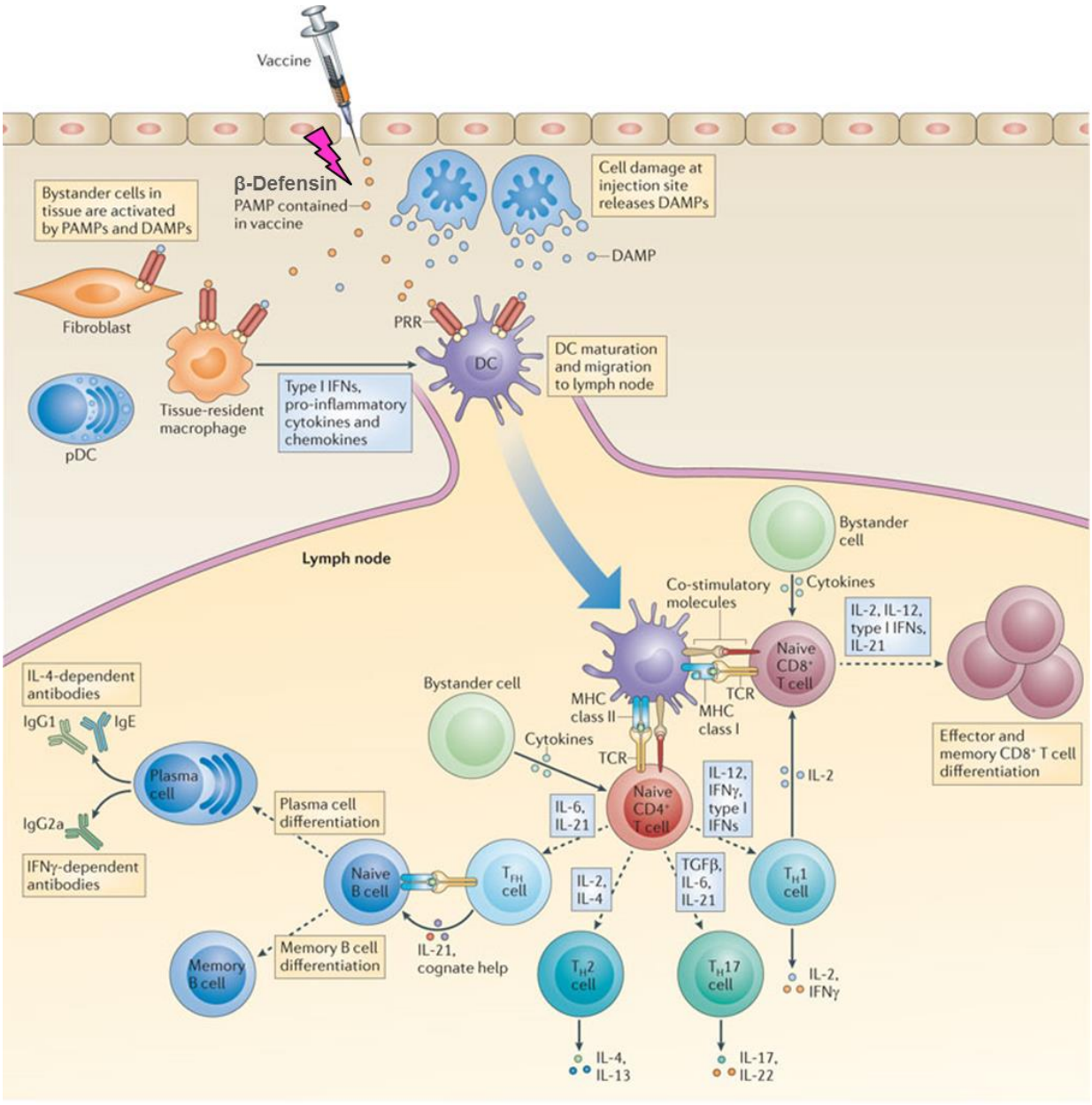

Figure 1.6 Enhancing immune responses with an iDC-attracting defensin in a DNA vaccine. Physical damage from delivery of the vaccine induces release of damage-associated molecular patterns (DAMPs) by resident cells. DNA vaccines, by their very nature, contain pathogen-associated molecular patterns (PAMPs), and the addition of β-defensin to the DNA increases the "danger signal" provided by the vaccine. These PAMPs and DAMPs are detected directly by pattern-recognition receptors (PRRs) and chemokine receptors expressed by dendritic cells (DCs), and stimulation of these encourages first in-migration of iDCs, followed by antigen uptake, DC activation, maturation and migration to the lymph nodes. The chemotactic effect of the defensin will encourage migration of greater numbers of pre-DCs and iDCs to the area and then the stimulatory effect of the defensin serves to induce maturation/activation and to orient and shape DC response. In the lymph nodes, the activated DCs present antigens with co-

stimulatory signals to prime naïve CD4+ and CD8+ T cells, and evoke their differentiation. CD4+ T cells differentiate into various T helper (Th) cell subtypes. Th cells may also acquire a T follicular helper (TFH) cell phenotype and foster the development of B cells to plasma cells or the germinal centre pathway. In addition, the cytokine expression profile of TFH cells can dictate B cell isotype switching. β-defensin containing DNA vaccines tend to promote IgG2a isotype antibody, indicative of a Th1 type response. Migratory DCs will also "pass" antigen to the resident splenic DCs that are very efficient at cross presentation. Depending on the balance between activating cytokines (Th1-derived IL-2 and other cytokines—see Figure 1.3 legend), activated CD8+ T cells differentiate into effector and memory CD8+ T cells. (Adapted by permission from MacMillan Publishers Ltd: Nature Reviews Immunology (Desmet and Ishii, 2012) [284]).

β-defensin and consequently acquire the vaccine antigen. The chances of acquisition of plasmid from transfected and/or dying skin cells by iDC would also be increased. (2) By providing a danger signal to the surrounding cells [137], we would expect that β-defensin would trigger increased release of chemokines CCL3 (MIP-1α), CCL4 (MIP-1β), CCL20 (MIP-3α) and CXCL10 (IP-10) Since iDCs express receptors (CCR1, CCR5, and CCR6) for these chemokines, more of these cells could be attracted to the site where the vaccine has been placed and/or where the encoded antigen is being produced by resident skin cells [106, 137]. (3) As with responses of local cells to invading pathogens, β-defensin in the vaccine might be expected to trigger increased secretion of cytokines, granulocyte-macrophage colony-stimulating factor (GM-CSF) and pro-inflammatory cytokines such as IL-1 and tumor necrosis factor alpha (TNF-α) [108]. These cytokines encourage highly phagocytic iDCs to capture dying cells and released-antigen efficiently using phagocytosis, macropinocytosis and receptor-mediated endocytosis [131, 132]. (4) Capture of antigen along with the influence of cytokines in the local microenvironment serves to induce maturation of antigen-loaded DCs [106]. CpG motifs present in the backbone of the DNA vaccine plasmid also provide a stimulus for DC maturation [285]. Maturation down-regulates CCR1, CCR5 and CCR6 expression, and up-regulates expression of CCR7. Antigen is processed and DCs undergo phenotypic and functional changes that result in

their transition from antigen capturing to antigen processing and presenting cells [135]. During this time, expression of CCR7's ligand, CCL21 (SLC) on the endothelium of the afferent lymphatic system, and by the stromal cells in the T-cell zone, direct migration of the maturing DC out of the tissue at the periphery and to the LN [137].

In many cases however, there are insufficient signals in the vaccine for maturation of DCs to occur. Without expression of CCR7, insufficiently matured DCs cannot properly traffic to the LN and thus would be unable to undertake the next steps that lead to induction of an efficient antiviral immune response. As an agent of maturation, β-defensin at this step should ensure upregulation of CCR7 with subsequent trafficking of the DCs to the LN. (5) The "licensing" of DCs, which is mediated by pathogen-derived signals and characterized by expression of CCR7, is followed by full maturation of DCs which takes place in the LN and may also be driven by CCL19 (MIP3-β/ELC) and CCL21 (6Ckine/SLC) [286]. In the LN, mature antigen-presenting DCs stimulate naïve T-cells through up-regulation of their surface costimulatory molecules CD80 (B7.1), CD86 (B7.2), and CD40, which bind to CD28 and CD40L (CD154) respectively on T cells. Expression of costimulatory molecules is a critical step in the DC maturation process and is required for T cell recognition of the presented antigen and subsequent T cell-mediated terminal maturation of DCs, a process characterized by further up-regulation of co-stimulatory molecules and the release of IL-1 and IL-12 by DCs and IFN-γ by T cells [139]. At this step the positive effect of β-defensin in the vaccine would be to assure maturation of the DC. As detailed previously (section 1.2.5), in the induction of humoral responses, mature DCs activate naïve B cells to plasma cells, and stimulate CD40-activated memory B cells to IgG-secreting cells [162, 163]. Antigen-specific CD4 T-cells and antigen-specific B-cells interact with mature DCs, B-

cells proliferate and antibody is produced and secreted [164, 165]. Obviously, DC maturation is critical for the inductive steps of DNA vaccine-induced immunity as outlined above, but in addition, antigen-presentation by an un-matured DC is known to induce tolerance to the antigen; a response that would be completely undesirable. Thus β-defensin may act to both improve induction of appropriate antiviral immune response and prevent unintentional induction of tolerance to the vaccine antigen.

2. HYPOTHESIS AND OBJECTIVES

I hypothesized that attracting iDCs to the site of DNA vaccination with β-defensin would result in improved uptake, processing and presentation of tgD by DCs leading to strong humoral and cell-mediated immune responses that would subsequently protect cattle upon challenge with BoHV-1.

Objective 1: Assess the chemotactic activity of bovine β-defensins for iDCs (*in vitro* and *in vivo*) and select the most chemotactic.

Objective 2: Construct plasmids that express the chemotactic β-defensin(s) alone and in combination with the BoHV-1 antigen tgD. Assess the immune and protective responses induced by intradermal DNA vaccination of mice and cattle using the plasmid treatment(s) with the most chemotactic β-defensin.

Objective 3: Determine the potential of a BNBD3/DNA complexed vaccine to improve humoral immune responses of mice and maintain robust cell-mediated responses, and to assess the immunomodulatory effects of BNBD3 *in vitro* on murine bone marrow derived DCs.

3. THE SYNTHETIC PEPTIDES BOVINE ENTERIC ß-DEFENSIN (EBD), BOVINE NEUTROPHIL ß-DEFENSIN (BNBD) 9 AND BNBD3 ARE CHEMOTACTIC FOR IMMATURE BOVINE DENDRITIC CELLS

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Third Chapter Transition/Overview

In this chapter/manuscript I addressed the first objective of the project which was to assess the chemotactic activity of bovine β-defensins for iDCs (*in vitro* and *in vivo*) and select the most chemotactic. Bovine iDCs were generated from bovine monocytes and were defined phenotypically at day 3 of culture by the expression of DC-specific receptors, and functionally by phagocytosis, proliferation and chemotaxis assays. Fourteen of the sixteen known bovine β-defensins were correctly synthesized. Using chemotaxis assays I screened these synthetic peptides for their ability to attract bovine iDCs. Bovine iDCs were consistently and equally attracted to BNBD3, BNBD9 and bovine EBD *in vitro*. Of the three peptides, BNBD3 was selected as the best peptide to be used for the rest of the project because 1) it was the peptide with chemotactic activity for iDCs from all animals tested; 2) it had the greatest chemotactic activity for iDCs at the lowest concentrations; 3) it was the peptide with the least complicated coding sequence for the mature form of the peptide, thus it was straightforward to encode in the DNA construct (s); 4) it was one of the simplest peptides to synthesize and gave good yields of correctly folded peptide; and 5) it is the most abundant (60%) of the 13 BNBDs in the bovine neutrophil [219]. The *in vivo* activity of BNBD3 was verified by increased migration of $CD205^{+}$ DCs in response to ID injection of BNBD3 to the skin These novel findings into the chemotactic nature of bovine β-defensins are a first step into exploration of their use in new iDC-targeting vaccination strategies. The iDC-chemotactic bovine β-defensin, BNBD3, was used for the studies in the subsequent chapters.

3.1 Abstract

Human and murine immature DCs (iDCs) are highly efficient in antigen capture and processing, while as mature cells they present antigen and are potent initiators of cell-mediated immune responses. Consequently, iDCs are logical targets for vaccine antigens. Originally discovered for their antimicrobial activity, and thought of as strictly part of the innate immune system, studies with defensins such as human β (beta)-defensin 2 (hBD2) and murine β-defensin 2 (mBD2) have shown that they can function as chemo-attractant for iDCs and, in vaccination strategies, can enhance antigen-specific adaptive immune responses. Most studies to date have been conducted in mice. In contrast, little is known about defensins in cattle. To expand our understanding of the role of defensins in modulating immune responses in cattle, DCs were generated from bovine monocytes and the immature state of these bovine DCs was characterized phenotypically and through functional assays. By day three (DC3), bovine monocyte-derived DCs stained positively for DC-specific receptors CD1, CD80/86, CD205, DC-Lamp and MMR. When compared to conventional six-day DC cultures or DCs cultured for ten days with and without maturation factors, these DC3 were functionally at their most immature stage. Fourteen of the sixteen known bovine β-defensins were synthesized and the synthetic peptides were screened for their ability to attract bovine iDCs. Bovine DC3 were consistently attracted to BNBD3, an analog of BNBD3 (aBNBD3), BNBD9 and bovine EBD *in vitro* and to aBNBD3 *in vivo*. These results are the first to describe chemotactic ability of synthetic bovine β-defensins for immature bovine monocyte-derived DCs.

3.2 Introduction

Beta-defensins are small (3.5 - 4.5 kDa), cysteine-rich, cationic peptides characterized by an N-terminal α-helix, and six conserved cysteine residues that form three disulfide bonds, and are released upon stimulation by microbial invasion and inflammation [214, 287]. They are either made by or released from cells primarily located in skin or mucosa [183, 222], and thus are in a good position to alert and recruit the cells of the adaptive immune system [183]. Dendritic cells are antigen presenting cells that are unique in their ability to induce primary immune responses and subsequently establish immunological memory [104-106, 288]. Thus DCs are desirable targets to increase vaccine success (reviewed in ref. [289]). As immature cells they capture and process antigens. They are also mobile and selectively express receptors for inflammatory chemokines such as CCR1, CCR2, CCR5, CCR6 that direct them to sites of inflammation [290, 291]. Recruitment of iDCs followed by their maturation at the site of infection is deemed critical for induction of appropriate adaptive immune responses to a number of pathogens and to vaccination [183, 292]. Immature (but not mature) DCs derived from human bone marrow CD34+ DC progenitors are chemo-attracted by human β-defensin 2 (hBD2) [214]. Similarly, mBD2 is selectively chemotactic for iDCs generated from mouse bone marrow progenitor cells [217]. Currently it is known that hBD2 and mBD2 can attract cell types other than iDCs, and that other β-defensins share this ability. In addition to iDCs, hBD2 chemoattracts memory T-cells [214], activated neutrophils [232] and mast cells [231]. Recently, hBD1 was found to be chemotactic for human monocyte-derived iDCs [293]. Human β-defensin 3 and its mouse orthologue mBD14 were reported to chemoattract iDC-mimicking (CCR6-expressing) cells [294] and Mo [215] as were hBD2 and its mouse orthologue mBD4 [215, 295]. Since monocytes

do not express CCR6 it was subsequently shown that these β-defensins exert their chemotactic effect via a different G_i protein-coupled-receptor, namely CCR2 [215]. Both CCR2 and CCR6 are expressed by iDC [217, 296] so theoretically, migration of iDC to hBD2 could be mediated by both receptors. Audaciously, a new report suggests that migration of iDC to hBD2 may not be even be receptor-mediated or alternatively may be only partially receptor-mediated. Morgera et al. (2011) reported chemotactic activity of hBD2 due to hBD2-induced membrane variations that correlate with increased cellular motility [297]. They found poor correlation of chemotaxis to hBD2 with surface expression of CCR6 by human peripheral blood monocyte-derived DCs in contrast to highly correlated expression of CCR6 with chemotaxis to MIP3α, the natural ligand of CCR6. Since only CCR6 was investigated and iDC were not pre-treated with pertussis toxin to test for involvement of Gi-protein-coupled receptors, it is not known whether CCR2 may have been involved in the non-CCR6-mediated chemotaxis to hBD2, or whether cell membrane mechanisms are predominant or are only involved when CCR6 and or CCR2 are not present [297]. Monocyte chemotaxis to hBD2 is also controversial. With respect to hBD2, the persuasive findings of Rohrl et al. (2010) [215] are contradicted by the earlier finding of Yang et al. (1999) [214] who reported that neither native hBD2 from psoriatic skin, nor recombinant or synthetically produced hBD2 were chemotactic for human peripheral blood monocytes. An explanation for this discrepancy was not found in the more current publication [215]. Whereas the *in vivo* observations of Vanbervliet et al. (2002) suggested that pre-DC and DC would be recruited to a site of infection through the orderly and sequential action of different chemokines via different receptors [298], this finding of Rohrl et al. (2010a) would suggest that the same chemotactic peptide (hBD2) could act on pre-DCs, DCs and many other types of cells due to the peptides` dual-receptor activity. Once recruited, β-defensin also has the ability to influence and

augment the immune response through activation and maturation of iDCs. Enhancement by β-defensin of the adaptive immune response including upregulation of co-stimulatory molecules and CCR7, and an increased Th1-polarizing proinflammatory cytokine profile was observed when mouse iDCs were treated with mBD2 [216]. Similarly hBD1 induced activation and maturation of iDCs, which included expression of CD91, a multifunctional receptor that can facilitate cross-presentation of antigen to the endogenous class I pathway [299], and for which defensins are a known ligand [293].

Previous studies with bone marrow derived DCs of mice [217] and with DCs generated from human bone marrow $CD34^+$ cells [214], have shown that, in a species-specific manner, β-defensin 2 is highly chemotactic for iDCs through chemokine receptor CCR6. Biragyn et al. hypothesized that via CCR6, the chemo-attractive nature of β-defensins could be utilized to both attract iDCs and encourage uptake of antigen, and that this could be used as a strategy to improve effective adaptive immunity in response to DNA vaccination [217, 218]. Intradermal DNA immunization of mice with mBD2 fused to antigen induces mucosal CTL and high-affinity neutralizing antibodies to the HIV-1 envelope [218], and is effective in therapeutic treatment for cancerous tumors [217]. It is thought that production of β-defensin by the DNA-transfected cells creates a chemotactic gradient at the site of immunization that encourages migration of iDCs from the circulation to the tissue [217, 218]. Chemo-attraction between the iDC and the β-defensin may bring more iDCs into the area, making it more likely that the antigen of interest is taken up by iDCs. Also, β-defensin-antigen fusion peptides may target antigen to these cells resulting in improved uptake of the antigen of interest [217]. Production of β-defensin also provides a "danger signal" to the cells in the surrounding area, triggering local expression of pro-

inflammatory cytokines and chemokines [217, 235]. The influence of these substances on the iDC, together with antigen uptake and processing, induces effective maturation and subsequent migration of these "maturing" cells from the tissue to the lymph node where they present antigen to naïve T-cells [137]. Mature antigen presenting DCs are potent, and uniquely capable of initiating both humoral and cell-mediated primary immune responses through cross presentation of antigen on both MHC I and MHC II molecules [110, 114, 217]. Given the plethora of new information regarding the chemotactic effect of hBD2 on many different types of cells (above and reviewed in ref. [300]), one can hypothesize that the beneficial effect of defensin in these vaccination strategies may have been due also to the immunomodulatory contributions of in-migrating Mo, neutrophils, and mast cells.

Whether the influence of β-defensin in the micro-environment at the site of immunization is enough to adequately mature an iDC that has taken-up an otherwise non-antigenic peptide to initiate an appropriate immune response, or whether β-defensin needs to be delivered as a β-defensin-antigen fusion, is currently unclear. The earlier studies in mice led to the conclusion that a non-antigenic tumor antigen needed to be targeted directly to the iDCs by physical linkage to mBD2 in order to elicit a protective immune response [217]. The more recent evidence that hBD3 activates human monocytes and myeloid DCs in a Toll-like-receptor (TLR)1 and 2-dependent manner [237], and that mBD2 acts directly on iDCs as an endogenous ligand for TLR4 causing up-regulation of costimulatory molecules and maturation of DCs [216] would suggest that for optimal DC maturation, antigen and defensin should be closely associated.

While the reports of immunomodulatory effects of human and murine β-defensins are increasing, little is known about defensins in large animals beyond their direct antimicrobial action. This represents a gap in the knowledge base that is required to confidently transfer promising technology such as vaccination strategies from the mouse model to application in larger species including humans. The effectiveness of β-defensin in attracting DCs, and its ability to elicit a protective response (linked or unlinked to antigen) has not been determined in cattle. To begin addressing these questions, the objective of this work was to describe for the first time the chemotactic ability of chemically synthesized and oxidized versions of fourteen bovine-β defensins for phenotypically and functionally immature bovine monocyte-derived DCs.

3.3 Materials and Methods

3.3.1 Synthesis and in vitro antimicrobial activity of bovine β-defensins

Tracheal antimicrobial peptide (TAP) [199], lingual antimicrobial peptide (LAP) [200], enteric β-defensin (EBD) [201], and bovine neutrophil β-defensins 1 to 13 (BNBD1-13) [219], including 2 analogs of BNBD3, were chemically synthesized on a Pioneer solid-phase peptide synthesizer using Fmoc chemistry [301]. For pE-BNBD3, a peptide of 41 amino acids without the pyroglutamate was similarly synthesized, and the pyroglutamic acid residue was coupled on the bench. The coupling was monitored by ninhydrin analysis until completion of the reaction. All peptides were then isolated and purified by HPLC, with the purity and molecular weight of the peptides confirmed by matrix-assisted laser desorption/ionization (MALDI) –time of flight (TOF) mass spectrometry as has been extensively described [301]. The linear peptides were folded by oxidizing the cysteine residues to form three intramolecular disulfide bonds as described [301, 302]. The oxidized peptides were then purified by HPLC.

In order to determine whether folding had taken place, oxidized peptides were evaluated based on their MALDI data. With the exception of BNBD10 and BNBD11, MALDI data confirmed the appropriate loss of 6 mass units that indicated that the correct number of disulphide bridges (3) had formed. Peptides were assessed for antimicrobial activity against *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853) using an inhibition zone assay modified from the method described by Zasloff [303]. Similarly modified methods have been used by others to verify antimicrobial activity [304-306]. Briefly, bacteria were grown overnight at 37 °C in 5 ml of Luria-Bertani (LB) broth. A 50 µl aliquot of the overnight culture was then grown to mid-log phase by incubating the cultures in 5 ml of LB broth for approximately 2 h at 37 °C or until the absorbance at 600 nm was 0.115 absorbance units. Forty microliters of standardized bacterial culture was further diluted with 160 µl of LB and then spread on Mueller-Hinton agar. The inoculated plates were allowed to stand at room temperature (RT) for 10 min and then 10 µl of sterile distilled H_2O (ddH_2O) as the negative control or 10 µl of bovine β-defensin (1 mg/ml) was pipetted onto the inoculated plate(s). Plates were again allowed to stand for at least 15 min at RT in order to dry the samples. Plates were then incubated overnight at 37 °C. Antimicrobial activity was quantified by measuring the diameter of the circular clear zones on the bacterial growth, and expressed as mm of clearance. The linear peptides showed limited or no antimicrobial activity, whereas all of the oxidized bovine β-defensins with the exception of BNBD10 and BNBD11 were able to inhibit, to varying degrees, the growth of bacteria. Zones of clearance showing characteristic antimicrobial activity of oxidized bovine β-defensins are displayed in

Figure 3.1 to provide a visual reference for the antimicrobial assay.

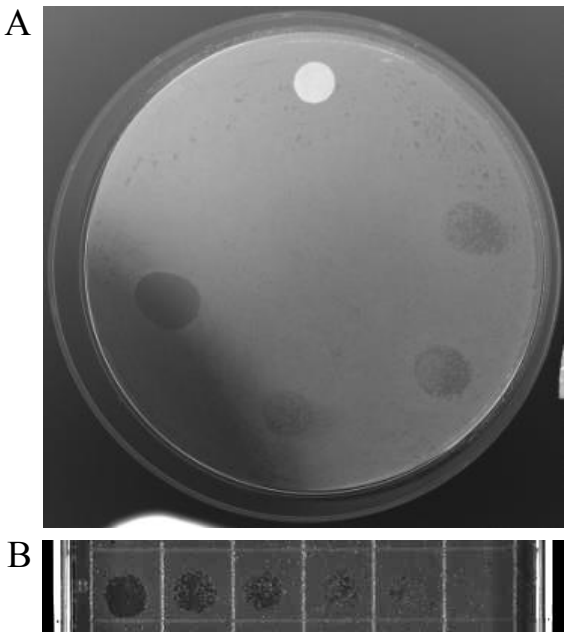

Figure 3.1 Inhibition zone assay for antimicrobial activity for sythesized bovine β-defensins. Sterile distilled H20 or synthesized bovine β-defensins (1 mg/ml) were pipetted (10 µl) onto *P. aeruginosa* inoculated plates as described. (A) Clockwise from the top: paper disk for plate orientation, TAP, LAP, EBD, aBNBD3, sterile distilled H20 center of the plate. (B) Descending dilutions of aBNBD3. From left to right: 1, 0.75, 0.5, 0.25, 0.1 and 0 mg/ml.

In conjunction with the MALDI-data, analysis of antimicrobial ability of the remaining fourteen peptides and the 2 analogs of BNBD3 suggest that appropriate disulfide bridges had formed although the connectivities of the disulfide bridges could not be unequivocally proven. Given that BNBD10 and BNBD11 were not correct based on MALDI-data, had no antimicrobial activity, and were subsequently found to be unable to chemoattract PBMCs, monocytes or DCs (data not shown), these two defensins were excluded from the rest of this work. Verification of

native disulfide connectivities was assessed by comparative HPLC of synthesized BNBD3 with native BNBD3 that was kindly provided by Dr. Micheal Selstead (University of California, Irvine, CA, United States)

3.3.2 Generation of bovine monocyte-derived dendritic cells

Peripheral blood mononuclear cells (PBMCs) were separated from heparinized (5 iu/ml) blood [307] by density gradient centrifugation at RT on Ficoll-Paque Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden, UK). Monocytes were isolated from PBMCs following incubation with 100 µl per10^8 PBMCs of anti-human CD14 paramagnetic particles (Miltenyi Biotech Inc., Auburn, CA, USA) and labelled cells were isolated from Midimacs LS columns (Miltenyi Biotech Inc.) according to the manufacturer's instructions. Monocytes were typically >97% pure by flow cytometry analysis and >99% viable by trypan blue exclusion. Cells were adjusted to 8 x 10^5 cells/ml in complete RPMI (cRPMI; RPMI 1640 [Gibco, Invitrogen Canada Inc., Burlington, ON, Canada] supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 µM non-essential amino acids, 1 mM sodium pyruvate, 50 µM 2-mercaptoethanol, 10 µM Hepes, and 50 µg/ml gentamycin). Supernatants from bovine IL-4 and bovine GM-CSF transfected chinese hamster ovary (CHO) cells (kindly provided by Merial Limited, Lyon, France) were added at previously determined optimal dilutions of 1/100, and 3 ml of cell suspension was added per well of six-well plates. Cells were incubated for up to 10 days at 37 °C in 5% CO_2 with one ml per well of fresh cRPMI and cytokines added every three days. DCs were harvested on days three (DC3), six (DC6) and ten (DC10) and evaluated by flow cytometry or used for functional assays. To mature DC10, a combination consisting of 100 ng/ml of recombinant human CD40 Ligand (Peprotech, Inc., Rocky Hill, NJ, USA), 100 ng/ml of

bovine TNF-α (R&D Systems Inc., Minneapolis, MN, USA) and 100 ng/ml of BNBD3 was added to cultures on day 7 or 8 and the subsequent "matured" cells were harvested on day 10. Preparatory work using these and other maturation factors, alone and in combination, showed that the above-described procedure resulted in DCs with increased stimulatory ability in an autologous antigen-specific lymphocyte proliferation assay (data not shown).

3.3.3 Phenotypic Analysis

***Staining of surface antigens*.** Monocytes, DCs, and responder lymphocytes used in proliferation assays, were washed in calcium-magnesium free phosphate-buffered saline (PBS; pH 7.2, Gibco, Invitrogen Canada Inc.) with 10% added bovine serum albumin (BSA; Fraction V, Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) and resuspended at not less than 5×10^6 cells/ml in cold fluorescence-activated cell sorting (FACs) buffer (PBS with 0.1% sodium azide and 10% FBS). Fifty microliters (µl) of cell suspension were added in 96-well plates to either 50 µl of FACs buffer plus FITC- or PE-conjugated specific primary monoclonal antibody (mab) for direct staining, or 50 µl of pre-diluted specific primary mab for indirect staining (Table 3.1) all at pre-determined optimal concentrations. Isotype-matched controls (Caltag/Invitrogen Canada Inc., Burlington, ON, Canada for indirect staining; BD Biosciences for direct staining) were used at 2-5 µl per well to detect non-specific staining. After 30 min on ice, cells were washed thoroughly and resuspended in 50 µl of FACs buffer. For indirect staining, bound mab was detected by adding 50 µl of 0.02 µg/ml FITC- or PE-labelled goat anti-mouse IgG1, IgG2a, IgG2b or IgM mab (Caltag/Invitrogen). After 10 min on ice, cells were washed, fixed with 2% formaldehyde and read on a FACScan flow cytometer (BD Biosciences, Mississauga, ON,

Canada). A minimum of 10,000 events was collected for each sample and flow cytometry data were analyzed with Cell Quest software (BD Biosciences).

Intracellular staining. Cells were fixed, permeablized and stained for intracellular expression of DC-LAMP, CD205 and mannose receptor-MMR (Table 3.1) using a BD Cytofix/Cytoperm fixation/permeablization kit (BD Biosciences) according to the manufacturer's instructions. Stained cells were fixed with 0.05% formaldehyde, read, and analyzed as above.

3.3.4 Uptake Assays

To compare the functional ability of Mo and DCs to take up antigen, 200 µl of 1 x 10^6 cells/ml in cRPMI were incubated at 37 °C (treatment) or on ice (control) with 180 µl of FITC-dextran (1 mg/ml in PBS) for 30 min [308, 309]. The cells were washed extensively with cold PBS, fixed with 2% formaldehyde and read as described above. Uptake of FITC-dextran was assessed by subtracting the fluorescence of the control cells from the fluorescence of the treatment cells.

Table 3.1 Monoclonal antibodies used for phenotypic analysis

Antibody	Isotype	Specificity[e]	Source[a]
MMIA	IgG1	CD3	VMRD
MCA1424G	IgG1	CD21	AbD Serotec
MM61A	IgG1	CD14	VMRD
IM3448	IgG1	hDC-LAMP[b]	Beckman Coulter
IM2741	IgG1	hCD206 (MMR) [b]	Beckman Coulter
CACT80C	IgG1	CD8	VMRD
130-091-242	IgG2a	hCD 14 [c]	Miltenyi Biotech Inc.
TH14B	IgG2a	MHC II	VMRD
TH97A	IgG2a	CD1 (CD1b)	VMRD
IDAC1323	IgG2a	hCTLA4 (CD80/86) [d]	ID Labs Inc.
MCA1651	IgG2b	CD 205	AbD Serotec
MCA1651F	IgG2b	CD 205-FITC	AbD Serotec
MCA 2041S	IgG2b	CD 172a	AbD Serotec
BAQ153A	IgM	CD11c	VMRD
CACT83B	IgM	CD4	VMRD

[a]VMRD, Veterinary Medical Research and Development, Pullman, WA, USA; BD Biosciences, Mississauga, ON, Canada; AbD Serotec, Oxford, UK; Beckman Coulter, Mississauga, ON, Canada; Miltenyi Biotech Inc., Auburn, CA, USA; ID Labs Inc., London, ON, Canada.
[b]Gliddon et al., 2004.
[c]Norimatsu et al., 2004.
[d]Pinchuk et al., 2003.
[e]Human-specific antibodies/ligands are identified by an "h". All others are bovine-specific.

3.3.5 Proliferation Assays

Immunization of cattle. Conventionally reared mixed-breed calves (~ 9 months old) were given a 2 ml subcutaneous (s.c.) immunization containing 50 µg of BHV-1 tgD [44, 310], 1 mg CpG oligonucleotide (ODN) 2007 (TCGTCGTTGTCGTTTTGTCGTT) supplied by Merial Limited (Lyon, France), and 30% Emulsigen (MVP Laboratories Inc., Omaha, NE, USA) in PBS (pH 7.4 Gibco, Invitrogen Canada Inc.). All animals were handled in accordance with the guidelines of the Canadian Council on Animal Care. After 14 days, sera from these animals were tested for tgD-specific IgG by ELISA as previously described [311]. Absorbance was read on a model Spectramax® 340 PC Microplate Spectrophotometer (Molecular Devices Corp., California, USA) at 405 nm, with a reference wavelength of 490 nm.

Preparation of responder lymphocytes. To compare the functional ability of Mo and DCs to present antigen, PBMCs from two animals with high IgG titres to tgD (bovine 39 and 84), six animals sensitized to tgD by one subcutaneous immunization (bovines 130, 132, 133, 134, 135, 137), and two tgD-seronegative animals, were separated from heparinized blood as described above. PBMCs were suspended at 5 x 10^7 cells/ml in fetal bovine serum (FBS) containing 10% DMSO [308], and stored in 1 ml aliquots at -70 C° for up to one month. On the day of the assay, PBMCs (5 x 10^7 cells) were thawed quickly in a 37 °C waterbath, washed in 30 ml of pre-warmed (37 °C) proliferation medium (PM; cRPMI with 1 ng/ml dexamethasone) and re-suspended in 45 ml of PM. PBMCs were depleted of Mo by plastic adhesion in a T-150 flask. After 2 h of incubation at 37 °C, non-adherent cells were carefully removed, washed and were resuspended at 1 x 10^6 cells/ml in PM. Responder lymphocytes typically had 1 to 4% contaminating Mo by FACs analysis and were at least 98% viable by trypan blue exclusion.

Preparation of APCs. Monocytes or DCs were suspended at 1 x 10^5 cells/ml in PM and 100 µl of antigen presenting cells (APCs) were dispensed in triplicate into round-bottom 96-well plates. APCs were pulsed in the presence or absence of 0.3 µg tgD for 4 h at 37 °C in 5% CO_2. Control wells were set up in the same plates in triplicate with 100 µl aliquots of medium alone (negative control), and medium plus antigen as described above to quantify proliferation of responder lymphocytes to antigen without added APCs. After 4 h, 100 µl of autologous responder lymphocytes prepared as described above were added to wells of the prepared plates. After 3 or 5 days of culture, cells were incubated with [methyl-^{3}H]thymidine (Amersham Biosciences, PQ, Canada) at a concentration of 37 Bq (1 µCi) per well for 16 h [312]. Plates were harvested with a Filtermate harvester and thymidine uptake was measured by scintillation counting with a TopCount NXT microplate scintillation counter (Packard Instrument Company, Meriden, CT, USA) [313].

3.3.6 Chemotaxis assays

Monocyte and DC chemotaxis to bovine β-defensins was performed using a 96-well disposable chemotaxis system with framed 5 µm polycarbonate membrane filters (ChemoTx system; Neuroprobe, Gaithersburg, MD, USA). The chemotaxis buffer (CB) used in these assays was PBS with $MgCl_2$ (1.2mM), KCl (5mM), $CaCl_2$ (5mM), glucose (5mM) and 0.1% BSA with an adjusted pH of 7.4 (personal communication; Dr. John Gordon, Dept of Vet. Micro., U. of S, Saskatchewan, Canada). Bovine β- defensins were diluted to 100, 10, and 1 ng/ml in CB and 29 µl was added to triplicate wells of the bottom chamber of the plate. Triplicate wells with just CB served as the control for non-specific migration as per the manufacturer's instructions. Monocytes and DCs suspended at 2 x 10^6 cells/ml were labelled with 5 µg/ml of Calcein AM

(Molecular Probes) for 30 min at 37 °C [314]. For pertussis toxin (PTX) treatments 1 x 10^6 cells/ml were incubated with 100 ng/ml of PTX (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) for 30 min at 37° C prior to labeling. Cells were washed and resuspended at 2 x 10^6 cells/ml in CM and 25 µl of this suspension was added to the top of the membrane above each filled well. After 90 min incubation at 37 °C, the non-migrated cells were removed from the top of the membrane by gentle vacuum, followed by wiping with a Q-tip, and gently washing the filter with PBS while holding the chamber at 45^o. The framed membrane filter was then removed from the rest of the chamber, inverted and placed in a 96-well multilabel plate reader (Victor 3V Multilabel Counter, PerkinElmer Life And Analytical Sciences, Inc., Woodbridge, ON, Canada). The plate reader was set in the top read position and cells on the bottom side of the filter were measured using the calcein fluorescence signal (excitation 485 nm; emission 535 nm). Chemotactic index (CI), defined as the fold increase of cell migration in the presence of test factors (directed migration) over cell migration in the presence of medium (random migration) [214], was calculated for each well by dividing the total fluorescence of the well by the mean fluorescence of the medium wells. A CI $\geq$ 2 is considered statistically significant ($p < 0.05$) [315]. For each experiment, 25 µl triplicate samples of fourfold-diluted cells were placed in bottom wells of the plate and read as described. These data provided a standard curve of the linear relationship and correlation between fluorescence and number of cells [314, 316]. We compared the automated method with the conventional method of Falk et al. [317] and established a standard curve for each time point in each experiment (data not shown). The standard curve for both Mo and DC3 showed a strong correlation between cell number and fluorescence as evidenced by r^2 values which were always greater than 0.99, validating this method.

3.3.7 Immunohistochemistry

Cryostat sections were allowed to air dry, fixed in -20 °C ethanol for 5 min, and then incubated for 2 h at RT with 5% horse serum and 5% FBS to block non-specific binding. Primary CD205-specific mabs (Table 3.1) or an isotype-matched control mab were added and the slides were incubated for 1 h at RT [318]. Slides were then washed three times with tris-buffered saline (TBS; 0.15 M Tris, 0.02 M NaCl; pH 7.6-7.8) containing 0.025% Triton X. The secondary biotinylated antibody (horse-anti-mouse IgG; Vector Laboratories (Canada) Inc., Burlington, ON, Canada) was diluted in TBS with 1% BSA and applied to the slides for 30 min. Slides were washed as above, whereupon the conjugate (ABC solution; Vector Laboratories (Canada) was applied for 30 min at RT. The slides were washed with TBS and the substrate (NovoRed; Vector Laboratories (Canada) Inc.) was added. Once optimal staining was achieved, the slides were washed with tap H_2O, counter-stained with modified Harris hematoxylin (Richard-Allan Scientific, Kalamazoo, MI, USA) for 30 sec, dehydrated and mounted in Cytoseal XYL (Richard-Allen Scientific).

3.3.8 Statistical analyses

Data were analyzed with the aid of Graphpad Prism 4.0 (San Diego, CA, USA) software. Differences between surface and intracellular antigen expression of Mo, DC3 and DC6, were analyzed by a two-way analysis of variance (ANOVA), followed by a Bonferroni *t*-test in case of a significant ANOVA. Differences were considered significant if $p<0.05$. Differences between uptake of FITC-dextran of Mo, DC3 and DC6 were analyzed by one-way ANOVA, followed by a Tukey's multiple comparison test when ANOVA indicated significant differences ($p<0.05$). Differences in proliferation of responder lymphocytes due to antigen or APC (zero or 10% Mo,

DC3, DC6) were analyzed by one-way ANOVA. When this test indicated significant differences ($p<0.05$), a Bonferroni *t*-test was used to compare differences between Mo, DC3 and where applicable, DC6. Differences were considered significant if $p<0.05$. Differences in CI of Mo, DC3 and where applicable, DC6 due to the effects of concentration or defensin was analyzed by two-way ANOVA. When this test indicated significant differences, a Bonferroni *t*-test was used to compare differences between defensins. Differences were considered significant if $p<0.05$.

3.4 Results

3.4.1 Phenotypic Characterization and Morphology of Bovine Monocyte-derived Dendritic Cells

Bovine CD14+ Mo were differentiated to DCs by culture with bovine cytokines GM-CSF and IL-4. By the third day of culture, plated Mo were non-adherent, and had the floating, rounded shape that is characteristic of human immature monocyte-derived DCs [319, 320]. Morphological changes that occur as Mo differentiate to DCs are shown in Figure 3.2A, and include an increase in overall size, a change in the shape of the nucleus, and the development of an extensive cytoplasm that gives the cells a veiled appearance. Dendritic processes were observed on some of the DC6, but not on DC3.

Monocytes and their subsequent DCs were stained with mabs specific for CD14, MHCII and CD11c, and for the DC-specific receptors CD1, CD80/86, CD205, DC-LAMP and MMR (mannose receptor) [308, 318, 321-325]. Since bovine DCs have not previously been described as immature or mature by increased or decreased expression of these receptors, and an immature phenotype was of most interest for use in the chemotaxis assay, receptor expression of DCs was

assessed on days 3 and 6, and for dendritic cell-lysosomal-associated membrane protein (DC-LAMP) and MMR, on day 10 as well. The numbers of cells expressing CD205, CD1, CD80/86 and MHC II increased from 2.5%, 1%, 0.5%, and 54% in the monocyte population, to 21%, 66%, 83% and 82% in the DC3 population, and then decreased to 13%, 60%, 75% and 75% in the DC6 population, respectively (Figure 3.2B). Differences between Mo and DC3 or DC6 were highly significant for CD1 and CD80/86 ($p<0.001$). The percentage of cells expressing MMR increased from 0.25% in Mo to 86% in DC3 ($p<0.001$) and 93% ($p<0.001$) in DC6. The percentage of cells expressing bovine CD11c was high in Mo at 99% and only decreased slightly to 93% by day 6. Intensity of CD11c staining, however, decreased significantly ($p<0.001$) from a mean fluorescent intensity (mfi) of 460 on Mo to a mfi of 173 on DC6 (data not shown), in good agreement with previous reports [309]. The population expressing CD14 detected by both human (h) CD14- and bovine (b) CD14-specifc mabs was high (96% and 98% respectively), confirming the purity of the CD14+ population at the start of culture.

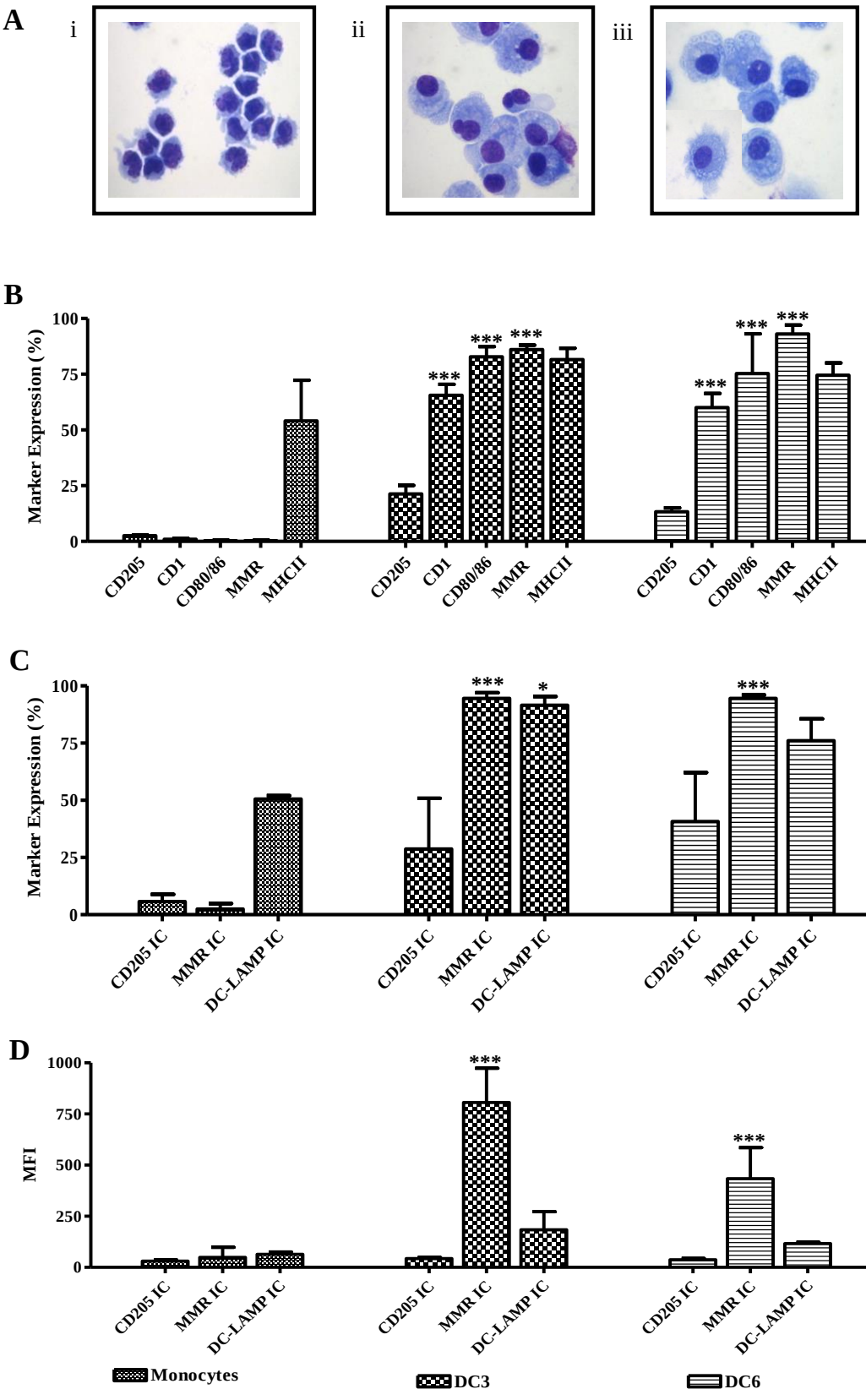
A
i
ii
iii
B
Marker Expression (%)
100
75
50
25
0
***
CD205
CD1
CD80/86
MMR
MHCII
C
Marker Expression (%)
*
CD205 IC
MMR IC
DC-LAMP IC
D
MFI
1000
750
500
250
0
Monocytes
DC3
DC6

Figure 3.2 Morphology and expression of cell surface and intracellular antigens of monocytes and monocyte-derived DCs. Pure populations (>98%) of monocytes were differentiated to DCs by culture in the presence of bovine GM-CSF and bovine IL-4 for 3 -6 days. (A) Cytospin preparations of peripheral blood CD14+ monocytes (Ai), and monocyte-derived DCs at day 3 (Aii) and day 6 (Aiii). Slides were dried and stained with Diff-Quick (Dade Behring, Newark, DE, USA). (B) Monocytes and DCs stained for expression of MHCII, CD1, CD80/86 (human CTLA4), MMR (human mannose receptor) and CD205. Changes in (C) percentage, and (D) mean fluorescent intensity (MFI) of cells expressing intracellular DC-specific antigens CD205, DC-LAMP (human CD208), and MMR. Stained cells were analyzed by flow cytometry. Experiments were conducted using bovines 39 and 84. Data are means $\pm$ sem of four independent experiments.

Staining with hCD14-specific mab decreased to 89%, and with bCD14-specific mab to 81%, in DC6 (data not shown). Intra-cellular expression of CD205, MMR and DC-LAMP (Figure 3.2C) in DCs was considerably higher than in Mo. This increase was significant for MMR ($p<0.001$) and DC-LAMP ($p<0.05$). Once cells had differentiated to DCs, there was little difference in the percentage (ie the number) of cells that expressed these receptors from day 3 to day 6 (Figure 3.2C) and to day 10 (data not shown). Mean fluorescent intensity (mfi) was also evaluated to determine whether differences could be seen in the quantity (amount) of receptor expressed by cells. Quantitatively, mfi of expression by DCs was increased over that of Mo for all the intracellular receptors, and was significantly higher for MMR ($p<0.001$) when DC3 were compared to Mo (Figure 3.2D). Also, a significant ($p<0.001$) reduction in mfi of MMR expression was observed from DC3 to DC6. Since maturation of DCs has been previously associated with a reduction in mfi of MMR [308, 309, 323, 326, 327], this, combined with the appearance of dendritic processes on cells at day 6, suggested that DC6 were more mature than DC3.

Next, four independent experiments were conducted wherein four animals were sampled in each experiment. Eight different animals were used in total. In good agreement with the earlier results, flow cytometric analysis of Mo and DC3 showed that for all animals in all experiments expression of the DC markers CD80/86, CD205 and MMR, by DC3, was consistent and highly significant (Figure 3.3). In these experiments detection of CD205 expression was much improved by direct staining with a fluorochrome-conjugated CD205. Minimal variation was observed with respect to expression of these DC-specific markers irrespective of animal (Figure 3.3).

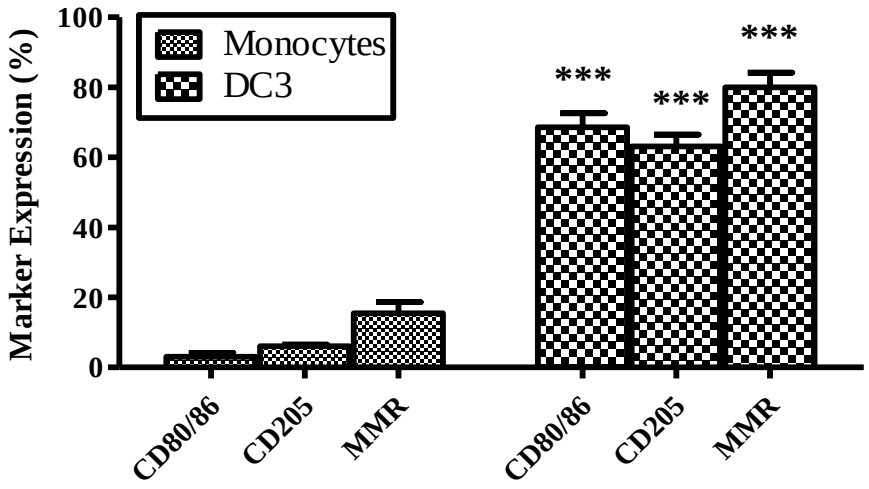

Figure 3.3 Direct staining of DC-specific antigens. Monocytes and DC3 from animals used in proliferation (**Figure 3.6**) and chemotaxis assays (**Figure 3.7**) were directly stained for surface expression of CD80/86, CD205 and MMR and analyzed by flow cytometry. Experiments were conducted using eight animals. Data are the means $\pm$ sd of at least four independent experiments where four animals were sampled in each experiment (n=16).

3.4.2 Functional Characterization of Bovine Monocyte-derived Dendritic Cells

3.4.2.1 Endocytic ability of monocytes and DCs

One of the main characteristics that set DCs apart from other APCs is their aptitude for antigen uptake [114, 135]. Since in studies with human DCs this greatly increases as Mo differentiate to DCs, peaks at immaturity, and then decreases as the cells mature [114, 135], functional characterization of bovine DCs included assessment of the cells' ability to take up FITC-dextran. The percentage of cells that took up FITC-dextran increased significantly as expected ($p<0.01$) from 35% in Mo to 91% in DC3 (Figure 3.4A).

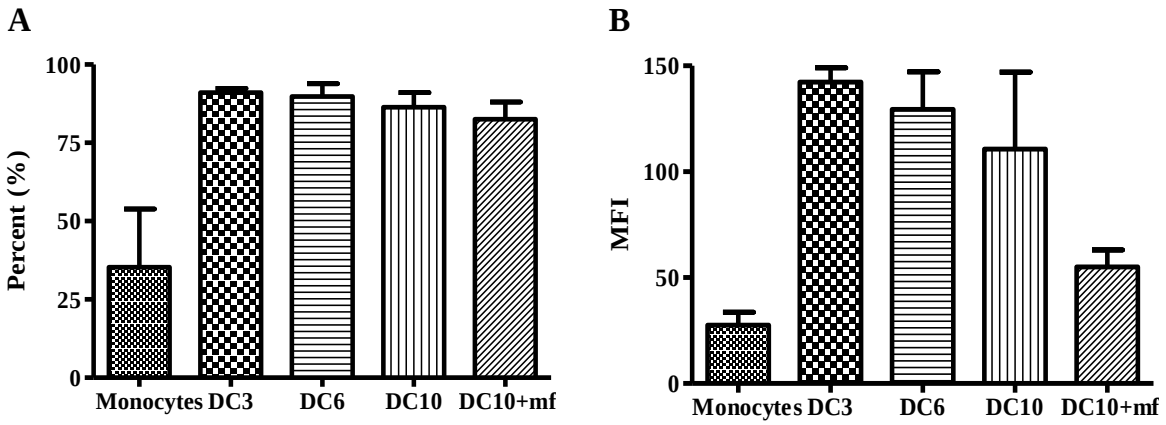

Figure 3.4 Uptake of FITC-dextran by monocytes, DC3, DC6 and DC10. Monocytes or DCs were incubated with FITC-dextran at 37 °C and on ice (control) for 30 min and then washed, fixed and analyzed by flow cytometry. Changes in uptake of dextran-FITC by monocytes and DCs are the values at 37 °C minus the values on ice (control) and are shown as both percentage (A), and fluorescent intensity (B). Experiments were conducted using bovines 39 and 84. Data are means $\pm$ sem of four independent experiments with the exception of DC10mf; data for which are from two independent experiments.

This only decreased slightly through days six to ten with little difference seen even in DC10 treated with a maturation cocktail composed of hrCD40L, bTNFα and BNBD3. Since the

percentage only defines the number of cells within a population that endocytose FITC-dextran, but does not quantitate the amount of FITC-dextran taken up by the cells, the relative maturation status of DCs was evaluated quantitatively by mfi. DC3 imbibed the largest quantity of FITC-dextran when compared to all other cells. Decreased mfi from day 3 to day 10 was observed with the lowest mfi by the matured DC10 (Figure 3.4B) as would be expected as DCs mature. Accordingly, these data show that DC3 have the highest capacity for antigen uptake, thus bovine DC3s are functionally immature at this stage of culture.

3.4.2.2 Antigen-specific proliferation of lymphocytes by monocytes and DCs

Along with the superior ability to take up and process antigen, DCs are the most capable of APCs at presenting antigen to, and stimulating proliferation of, lymphocytes [114, 135, 308, 309, 321, 323, 328, 329]. To ensure that our target cell population for chemotaxis to bovine β-defensins would be functional in this respect, autologous antigen-specific proliferation assays were performed with Mo and DCs as comparative APCs. Antigen-pulsed Mo or DCs were incubated with lymphocyte responder cells for three or five days. In agreement with other studies [330-332] overall proliferation was greater when cells were allowed to incubate for five days (data not shown). In subsequent 5-day proliferations, only DC3 and DC6, but not Mo, were able to significantly increase ($p<0.001$) proliferation above what was observed when responder cells alone were stimulated with tgD (Figure 3.5). DC3 and DC6 from bovine 39, and DC6 from bovine 84, were significantly better ($p<0.001$) at inducing proliferation of responder cells than were Mo (Figure 3.5). The greater capacity of DC6 to induce proliferation suggests that these cells are more mature than DC3.

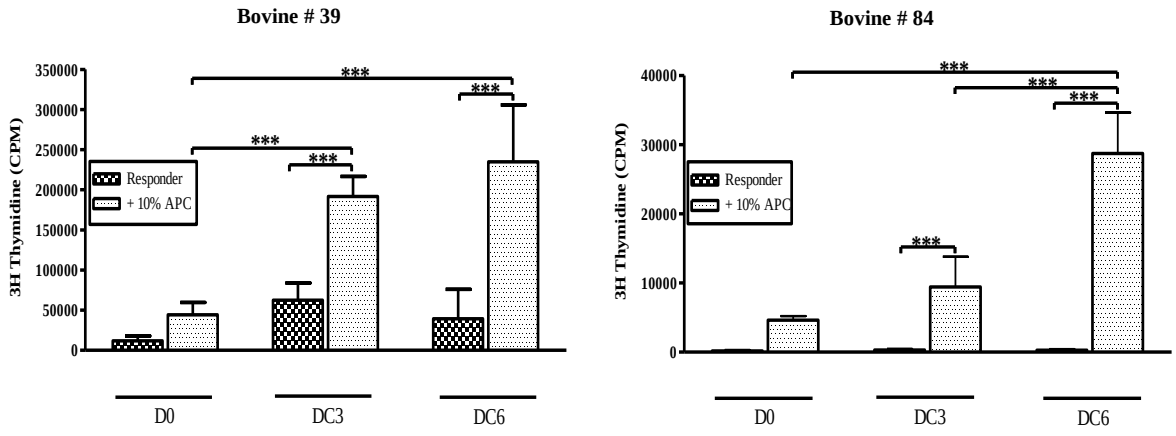

Figure 3.5 Proliferative responses of calves to tgD presented to autologous lymphocytes by monocytes, DC3 , and DC6. Autologous responder lymphocytes (10^5 cells/well) were incubated with or without monocytes or DCs (10^4 cells/well) in triplicate with tgD for 5 days. The animal # is indicated at the top of the graphs. Proliferative responses were measured by the incorporation of [methyl-^{3}H]thymidine . Data are mean CPMs $\pm$ SD of triplicate wells of two independent experiments with bovine #39 and two experiments with bovine #84. Significant differences between the ability of monocytes and DCs to induce proliferation of responder lymphocytes are indicated on the graphs where ***$p<0.001$.

To confirm these observations, six bovines previously exposed to BHV-1 were further sensitized to tgD. The tgD-specific IgG titres measured after 14 days ranged from 22,000 to 88,000 indicating that the animals had been adequately sensitized (data not shown). The two animals used as negative controls were confirmed BHV-1 negative. As described above, tgD-pulsed Mo or DC3 from these eight animals were incubated with

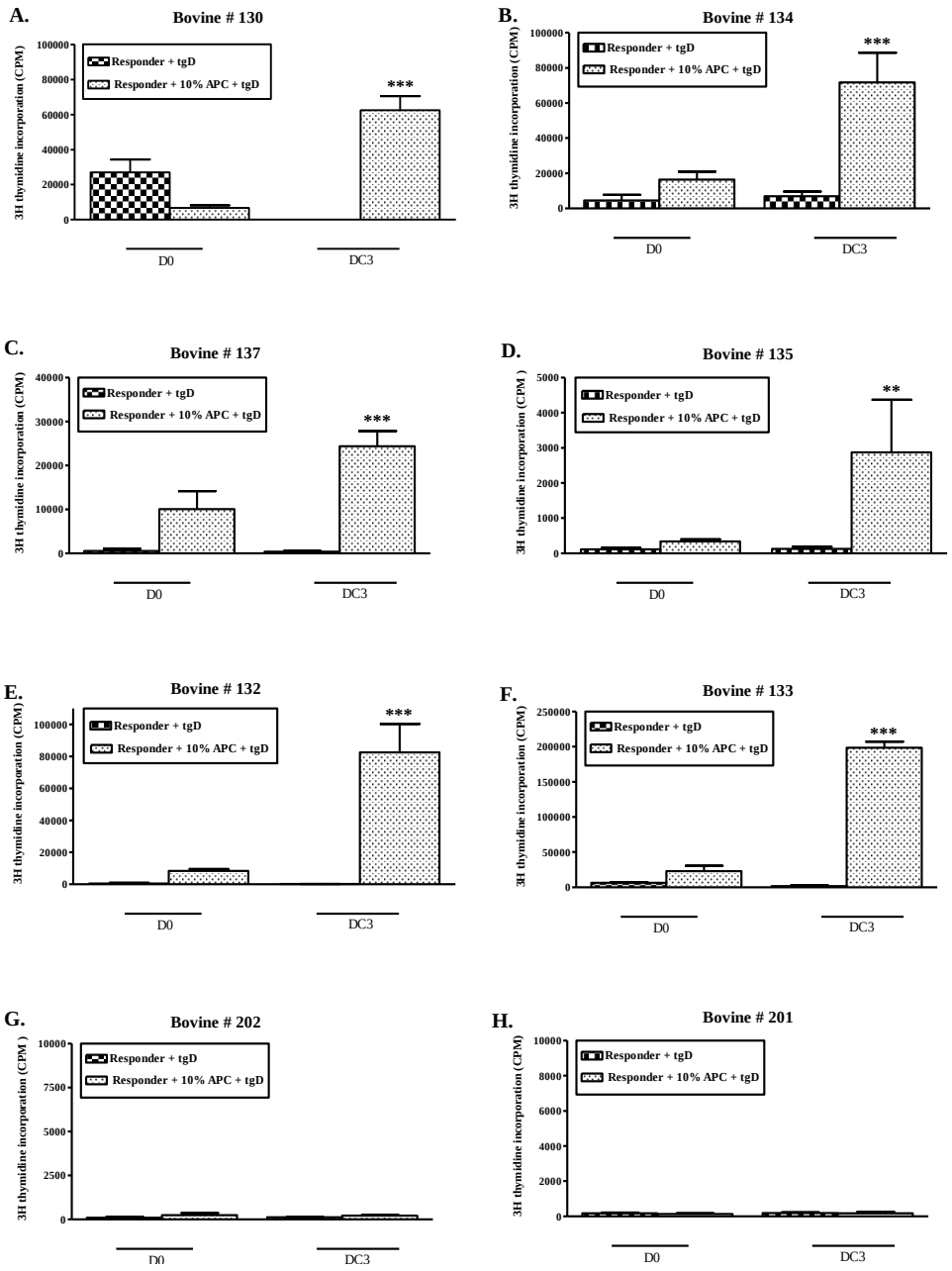

Figure 3.6 Proliferative responses of lymphocytes induced by autologous, tgD-pulsed monocytes and DC3 from six tgD-sensitized and two negative control donor animals. Responder lymphocytes (10^5 cells/well) from six sensitized (A-F) and two negative (G-H) animals were incubated with or without tgD, and with or without autologous tgD-pulsed monocytes or DC3 (10^4 cells/well) in triplicate for five days. The animal number is indicated for each panel. Proliferative responses were measured by the incorporation of [methyl-^{3}H]thymidine, and results are expressed as mean CPM $\pm$ SD of triplicate wells. Significant differences between the ability of monocytes and DCs to induce proliferation of responder lymphocytes are indicated on the graphs where **$p<0.01$ and ***$p<0.001$.

autologous lymphocyte responder cells for five days. Although we observed animal-to-animal variation in the magnitude of the proliferative response, DC3 from all sensitized animals were significantly more proficient (**$p<0.01$ and ***$p<0.001$) at inducing proliferation of responder cells than were Mo (Figure 3.6A-F), while no response (CPM<500) was seen in the negative animals (Figure 3.6G-H).

3.4.3 Chemotaxis of monocytes and DCs to bovine β-defensins

The concept of targeting vaccine antigens to DCs is rational as, of all APCs, DCs are the only ones that can stimulate naïve T-cells and induce an immune response to a previously unseen organism [104]. To determine whether bovine iDCs, like their human and murine counterparts [214, 217], would be attracted to the synthesized β-defensins, we used an in-vitro chemotaxis assay.

In the first set of experiments 14 peptides, including TAP, LAP, EBD, BNBD 1-9 and BNBD 12-13, were screened for chemotactic activity using a limited number of dilutions (0, 1, 10, 100 ng/ml). Data from four experiments with two donor animals were combined. To compare the magnitude of monocyte and DC chemotaxis, the arithmetic mean for each concentration was calculated. The arithmetic mean of the CI to all peptides at 0, 1, and 100 ng/ml, was 1.58, 1.45 and 1.33 for Mo; 7.43, 7.31 and 5.66 for DC3 and 4.08, 5.1 and 4.76 for DC6, respectively. Overall, we observed the lowest chemotaxis by β-defensins in the monocyte population; DC6 were more chemo-attracted than were Mo but less than DC3; and DC3 were the most chemo-attracted.

Since the DC3 population showed the greatest chemotactic activity, the DC3 data from each experiment were further examined to determine which peptide(s) had the best chemotactic potential for bovine iDC. The top seven β-defensins were first listed according to their "t" ratio and CI values. With the exception of BNBD9, defensins that were only active for one animal were excluded from the rest of the evaluation. The remaining six defensins, TAP, EBD, BNBD7, BNBD9, BND8, and aBNBD3 were then assigned a score of 7 (highest) to 1 (lowest) based on how they placed in terms of chemotactic ability for the DC3 in each experiment. For each defensin, these scores were totalled. The total score for each peptide was then divided by the number of experiments (4) to give an overall index ranking of chemotactic ability. BNBD3 and BNBD9 had the highest CI ranking of 3.5, closely followed by EBD at 3.25, and BNBD7 at 3. TAP ranked fifth with a CI ranking of 1.5. Based on these results, EBD, aBNBD3 and BNBD9 showed the most chemotactic potential and BNBD6 the least.

To test the validity of these results and confirm the chemotactic ability of EBD, aBNBD3 and BNBD9, the chemotaxis assay was repeated with a larger number of animals. For each of the eight animals tested in the proliferation assay, Mo and their respectively derived DC3 were assayed twice for chemotaxis to EBD, aBNBD3, and BNBD9. BNBD6, as the least chemotactic peptide, was included for comparison and as a control for the assay. Four independent experiments were conducted wherein 4 animals were sampled per experiment. When compared to Mo, DC3 were significantly more attracted to all concentrations of aBNBD3, BNBD9 and EBD; however, no differences were observed with BNBD6 (Figure 3.7). DC3 migration to all three concentrations of EBD, aBNBD3 and BNBD9, but not

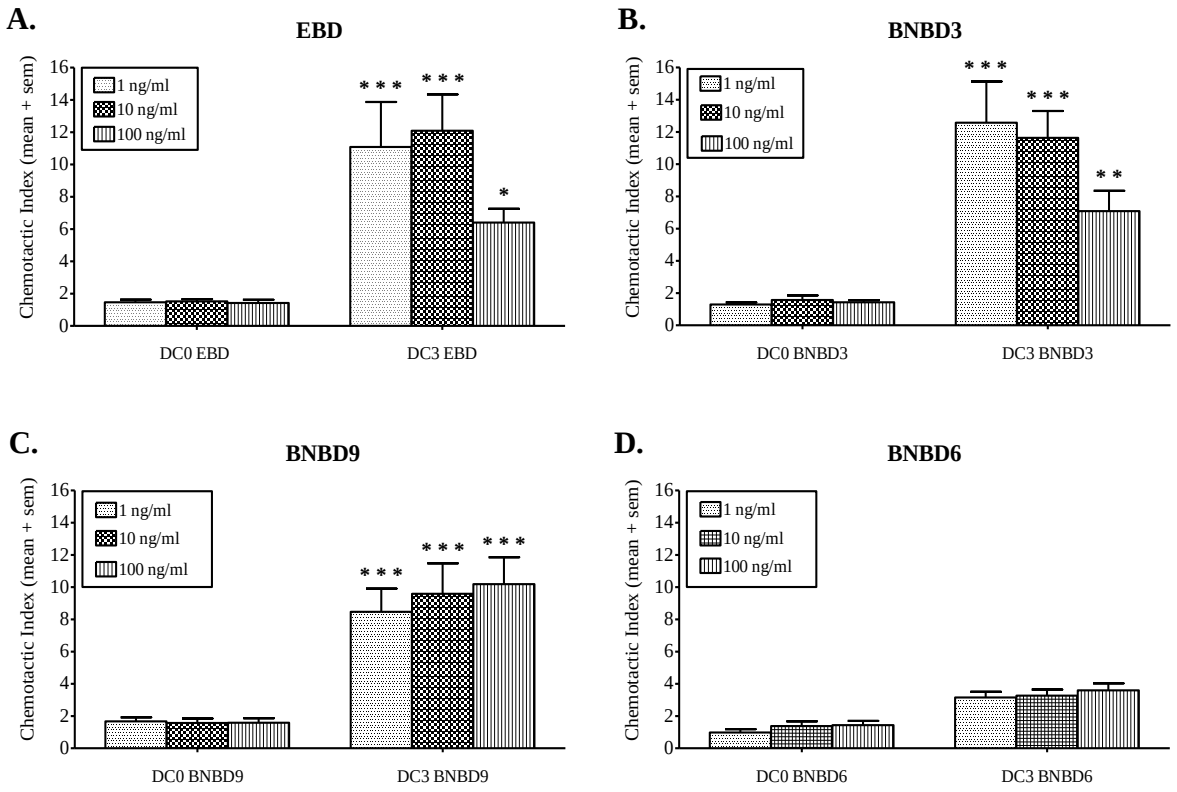

Figure 3.7 Chemotaxis of bovine monocytes (DC0) and DC3 to EBD, BNBD3 and BNBD9. Monocytes and DC3 were labelled with Calcein AM and placed on a 5 µm pore filter membrane above triplicate bottom wells filled with either medium or 1, 10 or 100 ng/ml of EBD, BNBD3, BNBD9 or BNBD6. Migration of cells toward medium or defensin was determined by reading the calcein fluorescence signal of migrated cells on the bottom of the filter after incubation at 37 °C for 90 min. Experiments were conducted using eight animals. Data are expressed as the chemotactic index (CI), and are shown as the mean values of the average CI $\pm$ sem of four independent experiments where four animals were sampled in each experiment (n=16). Significant differences between chemotaxis of monocytes and DC3 at each concentration are indicated on the graphs where *p<0.05, **p<0.01 and ***p<0.001.

BNBD6, was significantly greater than their migration to medium alone. As well, chemotaxis of DC3 to EBD and aBNBD3 at 1 ng/ml (**p<0.01 and ***p<0.001 respectively) and 10 ng/ml (***p<0.001 and **p<0.01 respectively) and to BNBD9 at 10 ng/ml and 100 ng/ml (*p<0.05) was significantly greater than DC3 migration to the same concentrations of BNBD6. As before,

chemotaxis by DC3 tended to be highest for EBD and aBNBD3 at the lower concentrations of 1 ng/ml and 10 ng/ml, and at these lower concentrations, was greater than chemotaxis to BNBD9 at any concentration. These results support the conclusion that DC3 are more chemo-attracted to β-defensin than are Mo and that chemotaxis of DC3 to BNBD3, EBD and BNBD9 was greater than to media or to BNBD6.

3.4.4 Verification of native disulfide connectivities of synthesized BNBD3

BNBD3 was one of the most chemotactic β-defensins, and thus was selected for further studies. Comparative HPLC and chemotaxis assays were performed to determine whether the cysteine pairing in synthesized BNBD3 occurred correctly during the oxidation process. BNBD3 peptides were synthesized and the amino acid sequences are shown in relation to EBD, BNBD9 and human and murine β-defensins (Figure 3.8). Sequences of the less chemotactic BNBD7, the poorly chemotactic BNBD2 and the least chemotactic peptide BNBD6 are also shown. Native BNBD3 purified from bovine neutrophils (kindly provided by Dr M.E. Selstead) conformed to the sequence given for pE-BNBD3, which has pyroglutamic acid, a modified residue of glutamine (Q), at the N-terminus. sBNBD3 conforms to the sequence for native and pE-BNBD3, but with the unmodified Q at the N-terminus and aBNBD3 is an analog with glycine (G) replacing Q at both the N-terminus and at position 27. MALDI-TOF analysis showed the correct mass of both the linear and

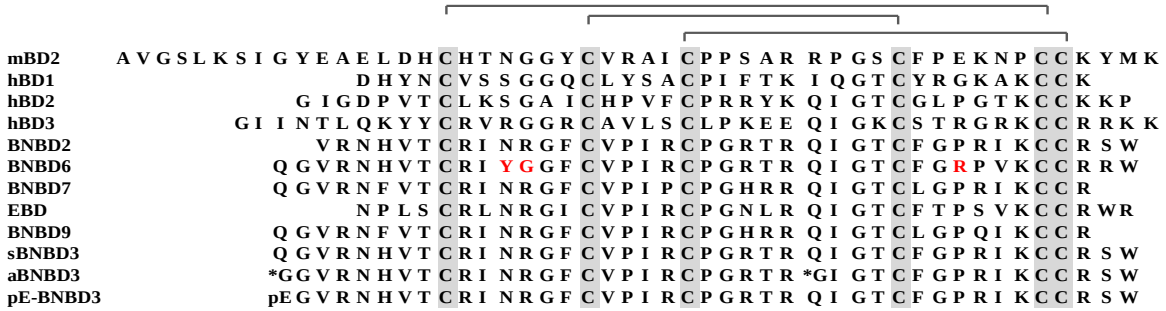

Figure 3.8 Amino acid sequence alignment of the synthesized bovine β-defensins BNBD2, BNBD6, BNBD7, EBD, BNBD9, sBNBD3 (published sequence UniprotKB/Swiss-Prot accession number P46161- Glutamine in positions 1 and 27), aBNBD3 (analog, Glycine [G] replaces Glutamine [Q] in positions 1 and 27), pE BNBD3 (pyroglutamic acid, the modified residue of Glutamine at N-terminus; the sequence of Native BNBD3), aligned with murine(m)BD2, human(h)BD1, hBD2 and hBD3. Aligned conserved cysteine residues are shown in shaded vertical bars and the β-defensin disulfide connectivities are shown at the top.

oxidised (folded) BNBD3s with a corresponding loss of 6 mass units during the folding reaction. Chromatograms of HPLC analyses showed the changes in the reaction products from the linear peptide at time 0 to the folded peptide at 72 hr during the oxidation of pE-BNBD (Figure 3.9A). The reaction product shifted initially to the left, but as the oxidation proceeded a defined peak appeared to the right. At 72 hr this defined peak was the dominating product, and thus most likely to be the correct form of pE-BNBD3. After 96 hr this fraction was isolated from the reaction mixture by HPLC. The analytical chromatogram (Figure 3.9B) shows that oxidized pE-BNBD3 elutes at a later time than the linear pE-BNBD3, showing evidence of change in structure during the oxidation process due to the disulphide bond formation. Most importantly, pE-BNBD3 and native BNBD3 co-eluted, and their retention time was increased from that of the linear pE-BNBD3 (Figure 3.9B). Since β-defensin disulphide bonding is characterized by cys1-cys5, cys2-cys4, and cys3-cys6 pairing, the native and synthesized (oxidized/folded) peptides only co-elute when analyzed by HPLC if they are identical [333, 334]. Accordingly, native and

pE-BNBD3 have identical amino acid sequences and disulphide connectivities thus authenticating correct folding under the oxidation conditions outlined. Since the native conformation usually favors that which is most thermodynamically stable [335, 336], the other synthetic peptides can be inferred to have the native conformation with the correct disulphide connectivities.

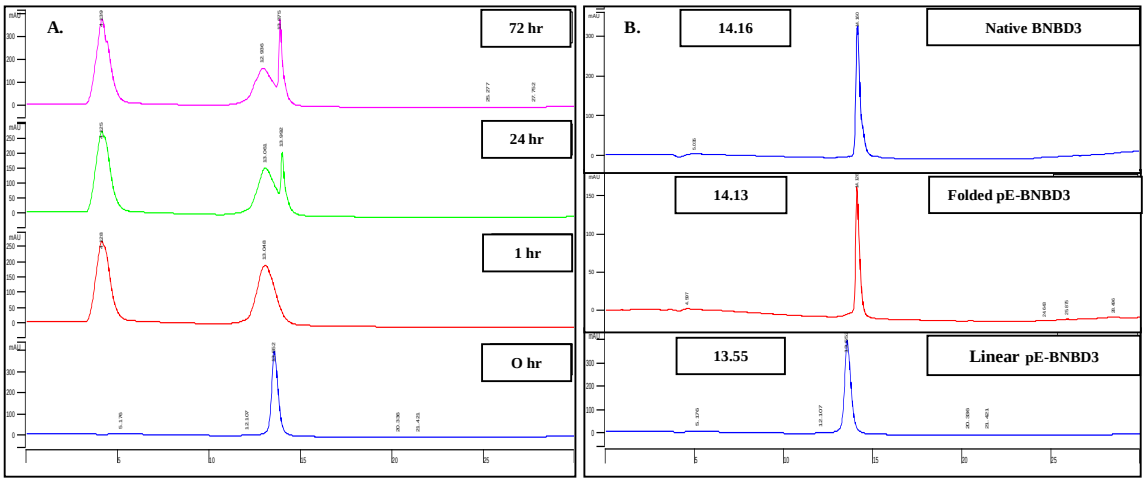

Figure 3.9 Oxidation of sythesized pE-BNBD3 and comparative HPLC of native BNBD3 and synthesized/oxidized pE-BNBD3. (A.) pE-BNBD3 oxidation reaction time course: from the bottom: Chromatograms of the change in the reaction profile beginning with the purified linear molecule at time 0 and after 1, 24, and 72 hrs. (B) from the bottom: chromatograms of the linear peptide, the synthesized/oxidized pE-BNBD3 after oxidation reaction of 96 h and the native peptide. Retention times for each are shown. The native and synthesized pE-BNBD3 co-elute.

In a chemotaxis assay, only peptides with the same connectivities have the same bell-shaped dose-response curves and peak migration at the same concentration. Chemotaxis to five 10-fold dilutions (0.001-1000ng/ml) of the native and synthesized BNBD3s was examined. All four peptides had similar bell-shaped dose-response curves and the concentration that induced the

maximum cell migration was the same, at 10 ng/ml. Migration of iDCs for all BNBD3s was significantly greater at 10 ng/ml ($p<0.001$) when compared to all other concentrations (Figure 3.10A). This strongly supports the contention that sBNBD3 and aBNBD3 have the same disulfide connectivities as pE-BNBD3 and native BNBD3. Importantly, this demonstrates that native BNBD3 is chemotactic for bovine iDCs while the three synthesized BNBD3s are equally chemotactic.

To determine whether the mechanism of migration of bovine iDCs to BNBD3 might be ligand-induced through G protein-coupled seven-transmembrane domain receptors, iDCs were treated with PTX (Figure 3.10A). No inhibitory effect of PTX on spontaneous migration of iDCs to CM was observed, thus PTX treatment did not affect migration by inhibiting cell motility. When compared to untreated cells; PTX pre-treament of iDCs completely abrogated migration to all BNBD3 peptides, and at 10 ng/ml this effect was highly significant ($p<0.001$). Thus, BNBD3 likely exerts its selective chemotactic effect on bovine iDCs through a Giα receptor(s).

Finally, checkerboard analysis was performed, with peptides at the optimized concentration of 10 ng/ml, to verify that migration of iDCs to BNBD3 was directed (chemotaxis) and not random (chemokinesis). A 10 ng/ml concentration of peptide below the filter induced

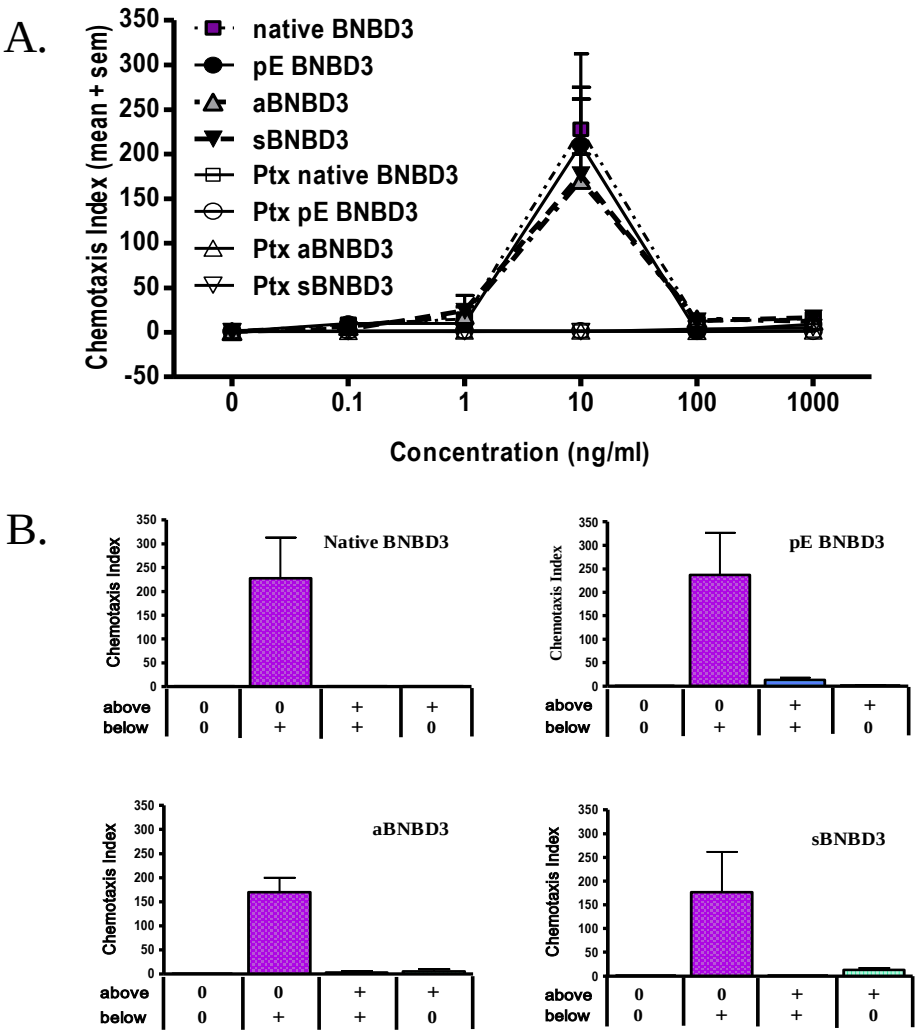

Figure 3.10 Chemotaxis of bovine DC3 to native and synthesized forms of BNBD3. DC3 were labelled with Calcein AM and migration of cells toward medium or defensin was determined. Data are expressed as the chemotactic index (CI), and are shown as the mean values of the average CI ± sem of four donor animals (n=4). (A) Migration of DC3 pretreated without (PTX-) or with 100 ng/ml of pertussis toxin (PTX) at 37°C for 30 min to native BNBD3, and the 3 synthesized BNBD3 peptides: pE BNBD3 (pyroglutamic acid at N-terminus), aBNBD3 (Glycine [G] replaces Glutamine [Q] in positions 1 and 27), sBNBD3 (published sequence Glutamine in positions 1 and 27). (B) For checkerboard analysis the optimal concentration (10 ng/ml) of each BNBD3 peptide was placed in the upper (above), lower (below), or both compartments of the chemotactic chamber. Migration of untreated cells to each peptide at 10 ng/ml placed in the lower compartment was significantly greater ($p<0.001$) when compared to all other concentrations, PTX-treated cells, and placement of peptide in the upper or both compartments.

significant ($p<0.001$) iDC migration, whereas an equal concentration of peptide above, or above and below the filter of the chemotaxis chamber did not increase cell migration over that to CM (Figure 3.10B). Since random migration was not observed with any of the peptides, migration of bovine iDCs to native BNBD3 and the synthesized BNBD3s is chemotactic and not chemokinetic.

In summary, the synthetic BNBD3 peptides, with pE-BNBD3 as an example, were properly folded with the correct disulphide linkages. Furthermore, the chemotactic ability of the peptides was dose-dependent with maximum migration at 10 ng/ml, and bovine iDC migration to the peptides was shown to be ligand-induced, chemotactic and not chemokinetic. We infer from these findings that the synthesized bovine β-defensins preferentially folded into a biologically active, native conformation.

3.4.5 BNBD3 increases migration of CD205+ cells with a DC-like morphology to the skin

To ensure that bovine β-defensin is not only chemotactic for DCs *in vitro*, but that it would also be chemotactic *in vivo*, punch biopsies (6 mm) were taken from untreated skin and at 3 h post-injection from bovine skin that had been injected ID with BNBD3 at 1mg/ml. As the biological activity of β-defensins may be reduced by suspension in ionic media such as saline or PBS [301], we first we established that the tissue is unaffected by injection of double distilled (ddH_2O) into skin and that the number of cells in the tissue was unchanged (data not shown). Subsequently, BNBD3 was suspended in ddH_2O and injected into the skin.

Cryostat sections from untreated and BNBD3-treated skin were probed with the mab to CD205, an isotype-matched control mab, or just the secondary antibody. When compared with sections

from an unvaccinated biopsy stained with mab to CD205 (Figure 3.11A) and sections from a BNBD3 biopsy stained with secondary antibody (Figure 3.11B) or with an isotype-matched primary plus secondary antibody (Figure 3.11B, Inset), sections from a BNBD3 biopsy stained with mab to CD205 showed more positively stained cells in the epidermis (Figure 3.11C-D), dermis (Figure 3.11C-D), and in the underlying lymph vessels (Figure 3.11C, E-F). At 100x magnification, CD205+ cells with DC-like morphology can be clearly seen in the dermis (Figure 3.11D). CD205+ cells lining the lymph vessels had a veiled morphology (Figure 3.11E), and when viewed at 250x magnification, appeared to be moving from the surrounding tissue into the vessel (Figure 3.11F). In other studies, CD205 mab stained more cells in the dermis and epidermis of bovine skin, with no apparent staining in lymph vessels [318]. We also observed higher numbers of CD205+ cells in the dermis and epidermis, and few in the lymph vessels, but only in sections of a biopsy taken 30 min post-injection with BNBD3 (not shown). These results suggest that in vivo chemotaxis by immature or pre-cursor DCs to BNBD3 occurs in 30 min to 3 h, and that the CD205+ cells observed in the sections from BNBD3 biopsies are, by 3 h post-injection, already migrating to lymph vessels and draining to the LN.

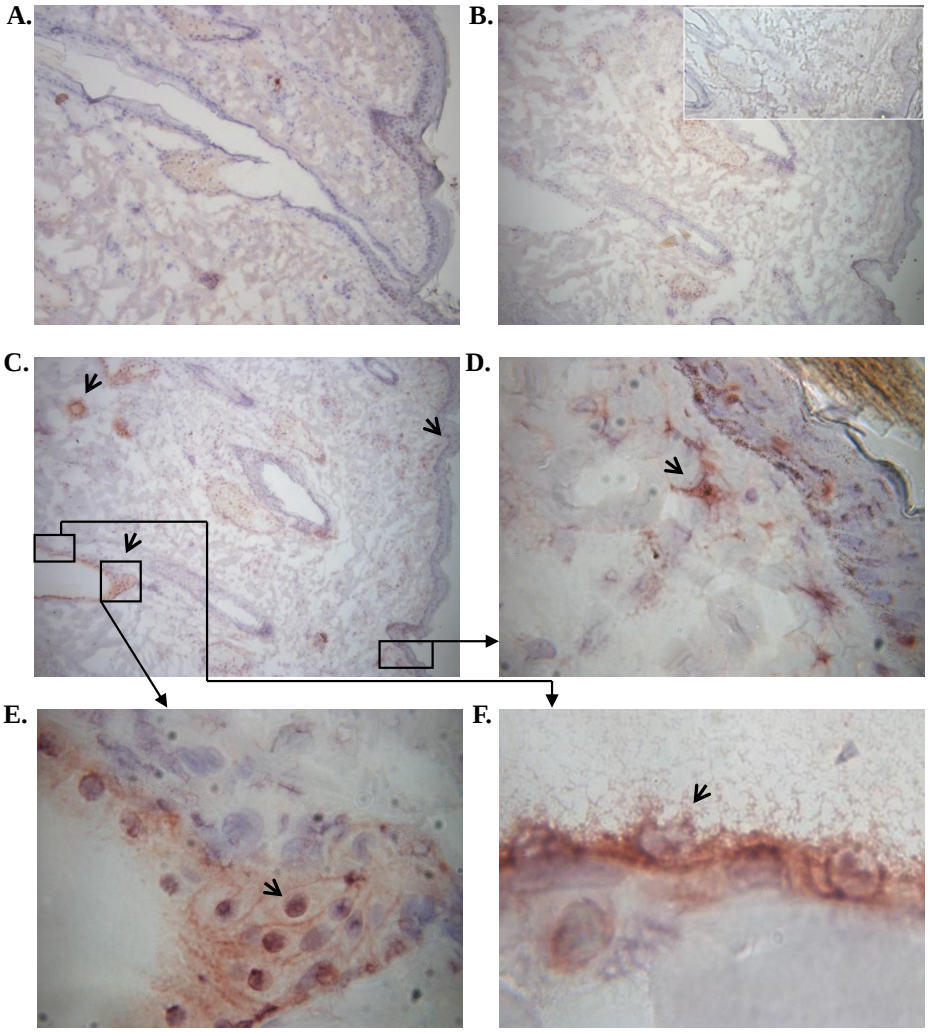

Figure 3.11 Migration of CD205+ cells 3 h post intradermal injection with BNBD3. Migration of CD205+ cells with DC-like morphology was assessed by immunohistochemistry using anti-CD205 mab MCA1651. (A) 10x magnification of a cryostat section from an unvaccinated biopsy probed with mab to CD205-MCA1651. (B) 10x magnification of a cryostat section from a BNBD3 biopsy probed with secondary antibody (Inset: staining with isotype-matched primary mab plus secondary.). (C) 10x magnification of a cryostat section from a BNBD3 vaccinated biopsy probed with mab to CD205-MCA1651. Stained cells can be seen in the epidermis, dermis and in the underlying lymph vessels; (D) 100x magnification showing cells with DC-like morphology in the dermis; (E) 100x magnification of stained cells lining the inner surface of a lymph vessel; (F) 250x magnification of cells with a veiled morphology and appearing to move from the surrounding tissue into the lymph vessel.

3.5 Discussion

Vaccination strategies for large, outbred animal species can be designed to take advantage of the unique APC characteristics of iDCs [275, 337]. Chemo-attracting immature bovine DCs to the site of antigen deposition by including a bovine β-defensin in its most effective form has the potential to improve the induction, magnitude and perhaps duration of adaptive immune responses to microbial and viral pathogens. Since little is known about bovine β-defensins, their potential for chemo-attracting bovine iDCs and the subsequent effect on the adaptive immune system of cattle, the objective of this study was to define for the first time the chemotactic ability of synthetic versions of 14 of the 16 known bovine β-defensins for immature bovine monocyte-derived DCs.

Since previous studies have shown that an immature phenotype and functionality is of importance in the ability of hBD2 and mBD2 to influence migration of iDCs and the induction of adaptive immune responses, we first performed a phenotypic and functional characterization of immature bovine monocyte-derived DCs. After three days in culture, the cells were morphologically different with a phenotype that was distinct from Mo and similar to human iDCs [319, 320], including expression of specialized antigen uptake receptors CD205, CD1, and MMR [326] which along with CD80/86 were virtually not expressed by Mo. Expression of CD14 and CD11c was high on Mo as would be expected in a pure positively selected population. Monocyte-derived bovine DCs are known to have higher expression of CD14, in contrast to human DCs or bovine DCs from other compartments [308, 309, 338, 339]. Thus, our observation that CD14 is expressed by bovine DCs does not exclude them as DCs. As well, when

compared to Mo, the intensity of CD11c expression by DCs was significantly reduced by day 6, in good agreement with previous reports of bovine monocyte-derived DCs [309]. The mab to human CD14 used to stain Mo and DCs for FACs analysis was the same as that used to isolate bovine Mo from PBMCs. Given that both Mo and monocyte-derived DCs stain with this antibody it is theoretically possible that a portion of the cells isolated from PBMCs as CD14+ Mo could be DCs or DC-precursors. This could explain why some Mo unexpectedly expressed DC-Lamp at low mfi. Thus the health status of the donor animal on the day of isolation may influence the makeup of the starting cell culture population, and explain some of the variability in phenotype and functionality seen in different experiments with different donor animals.

The mannose receptor is the major receptor for FITC-dextran [132] and highly expressed in iDCs. Because of its ability to repeatedly take-up and internalize FITC-dextran, and recyle to the cell surface, the MMR gives iDCs their hallmark endocytic characteristic and helps to functionally discriminate the iDC from Mo and more mature DC [132, 326]. We observed an increase in the number of cells that endocytosed and in the quantity of FITC-dextran taken-up by DC3 that was concurrent with increased surface and intra-cellular expression of MMR when compared to Mo. As the cells further differentiated to DC6 and DC10, the quantity of FITC-dextran taken up by the cells and the expression of MMR as evidenced by the decline in MFI, was reduced.

Maturation of DCs is also associated with an increase in costimulatory molecules with a concurrent increase in the ability to present antigen, as evidenced by the ability to stimulate resting lymphocytes [307, 308, 326, 331]. The expression of CD80/86 previously used to

establish differentiation of bovine Mo to DCs [309, 340] was increased. The DC3 from BHV-1 tgD sensitized animals were significantly more proficient at inducing proliferation of responder cells than were Mo, though there was variation between animals in the magnitude of the proliferative response. Animal to animal variation of cellular responses of outbred ruminant species is well documented [313, 341-343]. Typically this is attributed to MHC and cytokine gene polymorphisms in outbred populations [343], which could in turn influence the phenotype of circulating (responder) lymphocytes. Even in calves that share the same MHC haplotype, large differences in the magnitude of proliferative responses can be seen [341, 344]. As well, marked differences in innate immune responses of individual cattle [342] were observed, and this suggests that animal to animal variation cannot be solely attributed to MHC and cytokine gene polymorphisms of outbred species. Whether variation in the proliferative response is due to breed or animal differences attributable to the lymphocyte or DC populations [308], or whether it is influenced by differences in the innate immune responses is yet to be elucidated. Since our goal was to show that bovine iDCs, the target cell population for chemotaxis to bovine β-defensins, are capable of presenting antigen to, and stimulating proliferation of, lymphocytes, the significantly greater proliferation of autologous lymphocytes from sensitized animals when incubated with tgD-pulsed DC3 fulfilled this criterion.

Since antimicrobial activity is one of the most documented properties of defensins [345, 346], the peptides were tested prior to the chemotaxis assays. Because native bovine β-defensin peptides purified from neutrophil granules have proven bactericidal activity [219], to show that our synthesized peptides would be similarly biologically active, and to highlight any differences between linear and folded, both the linear and oxidized/folded forms of the synthesized bovine

beta-defensins were tested. When evaluated by plate-based assay, the linear peptides had limited or no antimicrobial activity. Additionally, the oxidized forms of BNBD10 and 11 that had not formed 3 disulfide bonds based on the MALDI data, were unable to inhibit bacterial growth. In good agreement with the findings of others, the oxidized peptides were salt-sensitive [175, 306, 346, 347]. Currently there are conflicting reports on whether synthesized peptides can be oxidized to achieve native disulfide pairing and thus conformation [348]. Also being questioned is the requirement for disulphide bonding and/or native connectivities [175, 349-351], or even whether the cysteines themselves are required for antimicrobial activity [346, 352, 353]. Whereas Wu et al. (2003) reported that linear hBD3 did not preferentially fold into its native conformation [334], and in fact oxidation gave rise to numerous oxidized species regardless of the 3 oxidation methods used, Harder et al. (2001) and Kluver et al. (2005), upon oxidation of the same 45 aa linear peptide, obtained a major product with the correct native connectivities [351, 354]. For the majority of the β-defensins including hBD1, hBD2, hBD27, mBD7, mBD8, BNBD2 and BNBD12 a straightforward oxidation of the hexathiol linear precursor peptides leads to the correct disulphide bonded isomer as the dominating product [346]. As with hBD1 and hBD2 [334, 355], oxidation of the synthesized linear bovine β-defensins using this method (with the exception of BNBD10 and 11) resulted in a dominant product that differed from the linear form by RP-HPLC analysis, and that could be easily purified. None of the peptides used in our studies gave rise to numerous forms. This is unlike hBD3, where oxidation yielded numerous species [334]. And although not a requirement, yields can be improved by utilization of a redox system such as the system used to fold the bovine β-defensin peptides in this study. In contraposition to the majority of the β-defensins, there is a small group that includes hBD3, hBD23, and hBD28, whereby a major species of correct peptide is not obtained by oxidative

folding [334, 356]. BNBD10 and 11 might also belong in this "difficult to oxidize correctly" group although confirmation would require further study. Wu et al (2003) found that non-natively folded forms of hBD3 had antimicrobial activity when evaluated by broth-dulution MIC assay [334]; whereas we found that oxidized BNBD10 and 11 had none/limited activity when evaluated by agar-plate assay. The contradiction in findings could be due to true differences in the peptides, or they could be a function of the qualitative and comparatively insensitive nature of the antimicrobial assay that was used in this investigation. With respect to antimicrobial activity of linear β-defensins, it has been reported in two reviews that for both bovine BNBD2 and 12, the presence or distribution of disulfide bonds is not essential for antimicrobial activity [346, 348]. Thus one might conclude that the linear BNBD2 and 12 were antimicrobial. However, for BNBD2, only results for the carboxy-terminal segment linear and oxidized peptides were reported [357]. Thus it appears that neither linear nor oxidized full-length BNBD2 was assessed for antimicrobial activity, or the results were not reported. For synthesized BNBD12, antimicrobial activity of oxidized BNBD12 was compared to and found to be less than that of the peptide with non-native conformation (C1-3, C2-4, C5-6) but comparison with the linear full-length BNBD12 was either not assessed or not reported [358]. Others have similarly reported no loss of antimicrobial activity by a linear hBD3 [334, 350]. However, the linear peptide tested was unlike ours in that the cysteines were replaced with alpha-aminobutyric acid (Abu). Whether this substitution contributed to antimicrobial activity is unknown. A linear hBD3 peptide containing six cysteines, that would be equivalent to the linear bovine beta-defensins in this study, was not tested [334, 350]. Importantly, like our synthesized bovine β-defensins, Routsias et al. (2010) found the linear form of hBD2 to be inactive and native disulfide connectivity essential for microbicidal activity of hBD2 [347]. Thus in the context of

the limited experimental data relating structure and antimicrobial activity, our finding that linear bovine beta-defensins are not antimicrobial whereas tri-disulfide folded peptides are, is not unreasonable.

Monocytes and DCs were compared in chemotaxis experiments, which we performed to answer whether bovine iDCs, like their human and murine counterparts [214, 217] are attracted to β-defensins, and if so, to which of the known bovine β-defensins they are most attracted In the initial screening, DCs were more chemo-attracted to bovine β-defensins than were Mo, and there was an overall trend for greatest chemotaxis to defensins in DC3 compared to DC6 population. Since maturation of DCs has been previously associated with lower chemotactic activity, this finding, combined with the reduction in mfi of MMR [132, 308, 309, 323, 326, 327], lower endocytic ability, increased CD80/86 expression, increased ability to stimulate lymphocytes, and the appearance of dendritic processes on DCs at day 6, suggests that DC6 are more mature and by inference, that DC3 are immature. Accordingly only DC3 were used in subsequent chemotaxis investigations as bovine iDC. Migration to defensin tended to be greatest at the lower concentrations and marginal at the higher concentrations. Although this effect was not statistically significant, chemo-attraction to the lower concentrations may be biologically significant, theoretically encouraging iDC to traffic from far-away to sites of injury, infection or vaccination in bovine skin, while protecting against influx of large numbers of iDC to highly inflamed areas due to their almost repulsion at higher concentrations.

aBNBD3, BNBD9 and EBD had the highest CI rankings, and also have comparatively high homology to hBD2, although none of the bovine β-defensins share greater than 50% sequence

homology with this human peptide. TAP, the bovine β-defensin with the greatest sequence homology to hBD2 ranked 5th and this was surprising as, like EBD and hBD2, it is an inducible epithelial defensin [185]. What makes one defensin more chemotactic than another, and what determines the kind of cell attracted, is currently unclear. Beta-defensins have a conserved tertiary-structure even though there is little primary structure or amino acid (aa) sequence similarity [306, 359, 360]. Only the six cysteines and two small residues (one is glycine, the other may be glycine or alanine) are highly conserved [333], although if chemically similar amino acids are included in the analysis, overall homology of β-defensins is increased [360]. In addition, the lack of connection between amino acid sequence and biological activity of the defensins has been well documented [345, 349]. Studies of sequence, structure and the effect (s) of changes on both with respect to defensin activity, have provided increased understanding of these relationships, but it is still not known if certain residues and/or structural features are required for defensin function [215, 333, 358]. Of the peptides that were tested in this study, BNBD6 was the least able to chemo-attract iDCs. BNBD6 has differences in 3 aa when compared to the sequences of the other bovine β-defensins (Fig 3.8, changed aa's shown in red letters) and these differences could account for its poor chemotactic performance. One of these is asparagine, an aa that is typically conserved in bovine β-defensins [227]. In BNBD6 asparagine, an aa with polar uncharged side chains, is substituted by tyrosine, an aa with a hydrophobic side chain, and a substitution that hypothetically could have unfavorable steric consequences. Further highlighting BNBD6's differences, Boniotto et al.(2003) reported a low level of homology between gallinacin, and BNBD6 with hBD3. Conversely, they did not find any homology (apart from the conserved residues) between hBD3 and any of the other bovine β-defensins, hBD1 or hBD2 [359]. BNBD2 was also poorly chemotactic for iDCs, and yet it is identical in sequence to

nBNBD3 with the exception that it is lacking the first 2 N-terminal amino acids. This change at the N-terminus could be responsible for its poor chemotactic activity compared to the highly chemotactic nBNBD3. Notable reductions in chemotactic activity, particularly CCR6-mediated chemotaxis, have been associated with changes to the N-terminal amino acids for hBD1 [361], and a truncated artificial β-defensin [362]. It is also possible that at least 4 residues preceding Cys^1 are needed at the N-terminus [355, 356, 362, 363]. BNBD7 was chemotactic for DC3 but less so than the top three. BNBD7 had a ranking of 3 that put it just behind EBD (3.25). The sequence of BNBD7 is the same as BNBD9 except BNBD7, like BNBD3, has an arginine (R) instead of glutamine (Q) residue at the third amino acid position preceding Cys^5. Why this single amino acid change would reduce chemotactic activity is not clear. In human defensin 5, the presence of arginine residues was found to improve host cell interaction presumably as a function of positive charge and hydrophobicity [364]. It may be that the change from the smaller uncharged glutamine (BNBD9) to the larger positively charged arginine (BNBD7) interferes sterically with a preferred or more stable conformation [333], particularly since this occurs in combination with a C-terminus that is relatively short (only 1 aa after Cys^6). The single amino acid change could also be responsible for the slightly lower chemotaxis of BNBD7 as compared to BNBD9, if it results in a peptide with looser structure that is less stable in media containing serum. The looser structure of hBD3 has been attributed to the presence of an argenine residue [359]; in turn, this structural characteristic has been correlated with increased degradation of hBD3 in serum [362].

To confirm our initial chemotaxis results, migration of Mo and DC3 to the three top β-defensins aBNBD3, BNBD9 and EBD was repeated with additional animals and compared to BNBD6.

Again DC3 were significantly more chemo-attracted to BNBD3, EBD and BNBD9 than Mo. Thus our earlier results were confirmed in these larger studies. The poor chemotaxis of bovine Mo to bovine β-defensins that we observed is contradictory to the findings of Rohrl et al. (2010a) who reported that hBD2 was chemotactic for human Mo, but in good agreement with the findings of Yang et. al (1999) who observed that hBD2 was not chemotactic for Mo. Appropriate controls were used in all studies, thus excluding many reasons for this discrepancy. One possible explanation could be the effect of the isolation method on subsequent monocyte function. In this and in the report by Yang et al. Mo were isolated from peripheral blood by Ficoll-Paque density gradient followed by immune-separation through columns, whereas Rohrl et al. (2010) used a Percoll gradient for the second step of the isolation. Activation of Mo as a result of being isolated over a Percoll gradient has been reported [365, 366] and this or perhaps variances in the surface receptors of the resulting monocyte populations [366] may account for the discrepancy between studies. Interestingly, we found that bovine Mo isolated using a Percoll gradient were unable to differentiate to DCs although whether this was due to activation or some other reason was not ascertained. Our chemotaxis results suggest that whatever method, be it a mechanism or receptor (s), is responsible for the ability of bovine iDCs to migrate to bovine β-defensins that these methods are not included in the monocyte repertoire. When chemotaxis of just the DC3 to aBNBD3, EBD, BNBD9 and BNBD6 was compared, highly significant differences were observed that suggested a slight chemotactic advantage of aBNBD3 and EBD.

Chemotaxis of iDCs to three synthesized forms of BNBD3s was also compared with chemotaxis to native BNBD3. We first established by RP-HPLC that the synthesis method we used would yield correctly folded (oxidized) peptides with the defined β-defensin disulphide connectivity.

Chromatographically, our synthesized N-terminal pyroglutamate (pE)-BNBD3 co-eluted with the native BNBD3 thus authenticating correct folding under the conditions outlined [333, 334]. Oxidation of the synthesized linear bovine β-defensins using this method resulted in a dominant product that differed from the linear form by RP-HPLC analysis, and that could be easily purified. This is unlike hBD3, where oxidation yielded numerous species [334]. None of the peptides gave rise to numerous forms, and the native conformation usually favors that which is most thermo-dynamically stable [335, 336]. It follows that the rest of our synthesized bovine β-defensins would also adhere to the native conformation with the correct disulphide connectivities, although we cannot prove this unambiguously. Chemotaxis activity offers another method by which to further confirm correct disulfide connectivity. Others have shown that analogs with non-native disulfide connectivities show peak chemotactic activity at different concentrations than peptides with the native conformation [334, 361, 367]. It follows that synthesized peptides with native disulfide connectivities most likely have the greatest chemotactic activity at the same concentration as the native peptide. We observed characteristic bell-shaped dose response curves [214, 306, 315, 361] with the same peak migration of 10 ng/ml for nBNBD3 and the three synthesized BNBD3 peptides. Thus synthesized pE-BNBD3 and native BNBD3 were biochemically identical based on amino acid sequence, mass spectrometry, and HPLC; and were biologically identical as shown by chemotaxis assay [368]. These chemotaxis results strongly suggest that all three synthesized BNBD3 peptides have the native conformation and show that all are equally chemotactic for bovine iDCs. This means that aBNBD3, which is the simplest peptide to synthesize with the highest yields, can be used to chemo-attract bovine iDCs as equivalent to nBNBD3. Directed migration of iDC to specific sites in tissue is also important as a mechanism in induction of appropriate specific immune response

[298, 369]. Using checkerboard analysis and pretreatment of iDCs with PTX, we determined that nBNBD3 and the three synthetic BNBD3s are directly and specifically chemoattractant for bovine iDCs. All BNBD3s induced iDC chemotaxis (directed migration) as opposed to chemokinesis (random migration). Although the bovine iDC receptors for BNBD3 are currently unknown, the PTX inhibition of bovine iDC chemotaxis indicates that this mechanism involves at least one seven-transmembrane Giα protein coupled receptor [315] without excluding the possibility of other receptors or mechanisms (described in introduction). As bovine chemokine receptors are discovered, and antibodies to these receptors become commercially available, further investigation can be done to identify the specific receptor(s) and or mechanism(s) involved in bovine iDC migration to bovine β-defensins.

In vivo chemotaxis by bovine DCs was determined by staining sections of bovine skin with mab to CD205 3 h after intradermal injection of aBNBD3. Some staining of CD205+ cells was evident in sections from un-vaccinated skin as would be expected in a steady state of in- and out-migration of DCs in normal skin [370]. More positively stained cells could be seen in the epidermis, dermis, and particularly in the underlying lymph vessels of sections from the BNBD3-treated skin. Other authors have shown that CD205 is expressed at high levels on DCs in the afferent lymph draining from the skin [308, 323, 371, 372], so we would expect that at later time periods, more matured DCs would be moving out of the tissues and into the lymph vessels. It is theoretically possible that injection of BNBD3 resulted in not only increased in-migration of DCs and DC-precursors from the circulation, but that it also induced migration of resident DCs to the site of injection [373] followed by their subsequent movement to the underlying lymph vessels. This suggests that *in vivo* chemotaxis by immature or pre-cursor DCs

to BNBD3 is a relatively rapid response that can take place in under 3 h. These results corroborate and support our in vitro results that BNBD3 is chemotactic for immature bovine DCs.

In conclusion, we defined phenotypically and functionally, the bovine iDC. We demonstrated that bovine iDCs are chemo-attracted by β-defensins and that BNBD3, BNBD9, and EBD are the most chemotactic. These new findings into the chemotactic nature of bovine β-defensins are a first step into exploration of their use in new iDC-targeting vaccination strategies.

4. INCLUSION OF THE BOVINE NEUTROPHIL BETA-DEFENSIN 3 WITH GLYCOPROTEIN D OF BOVINE HERPESVIRUS-1 IN A DNA VACCINE MODULATES IMMUNE RESPONSES OF MICE AND CATTLE

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Fourth Chapter Transition/Overview

In this chapter/manuscript I built on the work of the previous chapter 3 and addressed the second objective of the project which was to 1) construct plasmids that expressed the iDC chemotactic β-defensin, BNBD3, alone and in combination with the BoHV-1 antigen tgD, and 2) assess the immune and protective responses induced by intradermal DNA vaccination of mice and cattle using the plasmids encoding BNBD3. In mice, co-administration of BNBD3 on the separate plasmid enhanced the tgD-induced IFN-γ response, but not the antibody response. BNBD3 fused to tgD did not affect the antibody levels or the number of IFN-γ secreting cells, but increased the induction of tgD-specific CTLs. In cattle, BNBD3 delivered with a separate plasmid did not enhance immune responses, while the addition of BNBD3 as a fusion construct increased CMI but not humoral responses. Cattle were equally protected from BoHV-1 infection by DNA vaccines encoding tgD alone or as a fusion with BNBD3.

4.1 Abstract

Bovine herpesvirus-1 (BoHV-1) causes recurrent respiratory and genital infections in cattle, and predisposes them to lethal secondary infections. While modified-live and killed BoHV-1 vaccines exist, these are not without problems. Development of an effective DNA vaccine for BoHV-1 has the potential to address these issues. As a strategy to enhance DNA vaccine immunity, a plasmid encoding the bovine neutrophil beta defensin-3 (BNBD3) as a fusion with truncated glycoprotein D (tgD) and a mix of two plasmids encoding BNBD3 and tgD were tested in mice and cattle. In mice, co-administration of BNBD3 on the separate plasmid enhanced the tgD-induced IFN-γ response, but not the antibody response. BNBD3 fused to tgD did not affect the antibody levels or the number of IFN-γ secreting cells, but increased the induction of tgD-specific cytotoxic T lymphocytes (CTLs). In cattle, the addition of BNBD3 as a fusion construct also modified the immune response. While the IgG and virus neutralizing antibody levels were not affected, the number of IFN-γ secreting cells was increased; specifically the $CD8^+$/IFN-γ^+ T cells including $CD8^+$/IFN-γ^+/$CD25^+$ CTLs after BoHV-1 challenge. While reduced virus shedding, rectal temperature and weight loss were observed, the level of protection was comparable to that observed in pMASIA-tgD-vaccinated animals. These data show that co-administration of BNBD3 with a protective antigen as a fusion in a DNA vaccine strengthened the Th1 bias and increased cell-mediated immune responses, but did not enhance protection from BoHV-1 infection.

4.2 Introduction

Bovine herpesvirus-1 (BoHV-1) causes recurrent respiratory and genital infections in cattle. As the causative agent of IBR, BoHV-1 is one of several pathogens that interact, typically during times of stress, to cause respiratory disease and death in calves and feedlot cattle [70, 71]. Economic losses associated with BRDC, of which BoHV-1 is considered a major etiological agent, have been approximated at 640 million annually in the United States according to a report in 2000 [374]. According to a study performed in 2006 an economic loss of $13.90 per animal in the feedlot is estimated, due to lower gains and treatment costs for BRD [375]. In the breeding herd, BoHV-1 infection has been implicated in reproductive diseases, poor reproductive performance, and in abortion [376-379]. Costs and economic losses due to BoHV-1 infection and reactivation are thus observed in the breeding herd, but are difficult to calculate due to a lack of established data.

Immunization of cattle against BoHV-1 infection is currently achieved using either MLV or KV commercial vaccines [374]. The MLV vaccines are generally considered most effective as they stimulate both humoral and cell-mediated immunity [90, 278]. However, adverse effects associated with the use of MLV vaccines include latent infection and immune suppression [71, 90], and abortion in pregnant animals [378, 380]; the latter effect limiting the use of MLV vaccines in some groups of cattle, notably bred cows and heifers. Thus, KV vaccines may be recommended for use in the breeding herd as they are considered safer [381]. Additionally, although inactivated vaccines are non-infectious and are generally stable, they have the disadvantage of being poorly immunogenic (unable to stimulate cellular immunity) and typically

require adjuvants and/or several immunizations (reviewed in [382]). As neither type of vaccine fully protects cattle from BoHV-1 infection and both have inherent shortcomings [98, 276, 383], new vaccines are being sought that would be effective, safe in all groups of cattle, and economical.

DNA vaccines are one such type. DNA vaccines are cost-effective and can be designed, manufactured and stored with relative ease [241]. They also are non-infectious and do not promote inflammation at the site of immunization [241]. These are important factors in vaccines for food animals such as cattle [243], and in vaccination strategies for any virus for which the potential for reversion and spread of MLV vaccines is a major drawback. Furthermore, DNA vaccines can be used as marker vaccines to differentiate vaccinated and virus-exposed animals in eradication programs [243]. They also have the potential to initiate immunity in neonates born to immune mothers [244]. Similar to MLV vaccines and natural infection, and in a manner unique for a non-infectious moiety, DNA vaccines can induce MHC class I-restricted CD8 cytotoxic T lymphocyte (CTL) responses and produce neutralizing antibodies [246]. Since their discovery in 1990 [247], there have been over 600 reports of successful induction of immune responses in animals given DNA vaccines [249]. However, in the majority of these studies mice were used as an experimental model. In large animals much weaker responses have been observed which were not always sufficient to provide protective immunity [243]. To address this, several methods have been employed to increase the potency of DNA vaccines and/or manipulate the immune response including improving cellular delivery of plasmid DNA, increasing antigen production, and genetic adjuvanting [384-387].

One method of genetic adjuvanting that has shown promise in the mouse model [217, 218] and also in the chicken [388] to improve the potency and/or modulate the immune response to a DNA vaccine is to deliver antigen as a fusion with a beta (β)-defensin. Beta-defensins are cationic, membrane active, antimicrobial proteins of the innate immune system that participate in defense against microbial pathogens [199]. In cattle, as in mice, β-defensins are found primarily in skin or mucosal tissues, and they are released in response to infection or injury. This suggests that, in a manner similar to that shown by Yang et al. [183], they are ideally positioned to recruit the cells of the adaptive immune system and may serve to initiate and amplify the adaptive immune response to invading pathogens. As such, they are an attractive target for strategies aimed at enhancing the antigen-specific adaptive response to a DNA vaccine. The efficacy and/or immunomodulatory effect of a DNA vaccine designed to express β-defensin in a large animal model such as cattle has not been tested. Since CMI is important in protection from viral infections, and this has been shown in murine and chicken models to be enhanced by the inclusion of β-defensin as a fusion construct in a DNA vaccine, it is logical to think that a similar strategy may improve efficacy of a BoHV-1 DNA vaccine for cattle.

Here we describe for the first time a DNA vaccine encoding the mature form of the chemotactic bovine β-defensin-3 (BNBD3) peptide [389], either co-administered on a separate plasmid or fused with a truncated secreted form of glycoprotein D (tgD) of BoHV-1, a well-characterized vaccine antigen that has previously been shown to impart partial protection [44, 263] from BoHV-1 infection to cattle. BNBD3 was shown to modulate the type of tgD-specific immune response induced by DNA immunization, both in mice and in cattle.

4.3 Materials and Methods

4.3.1 Construction of plasmids

Expression plasmids encoding the mature form of the BNBD3 peptide (GenBank accession no. AF016396) were constructed according to the strategy shown schematically in Figure 1. BNBD3 was inserted into pMASIA and pMASIA-tgD [390] in three pieces (Figure 4.1). Complementary oligonucleotide pairs, shown in Table 4.1, were synthesized (Sigma-Aldrich, St. Louis, MO, USA) based on the published coding sequence for the mature BNBD3 peptide (GenBank accession no. AF016396). The first piece was synthesized with a 5' BamHI site (BNBD3-1), the second with 5' phosphorylation (BNBD3-2) and the third with a 3' HindIII site (BNBD3-3). BNBD3-3 was modified for insertion into pMASIA-tgD to create the defensin-viral antigen fusion construct BNBD3-tgD by removing the stop codon and adding the coding sequence for a defined octapeptide linker NDAQAPKS [391-393] to the 3' end of BNBD3-3 to generate BNBD3-3L. This octapeptide was used to connect the 3' carboxy end of the BNBD3 coding sequence to the 5' amino terminus of the sequence for tgD. The coding sequence for this linker has been described previously as 5'-AAC GAC GCA CAA GCA CCA AAA AGC/TCA-3' [392, 394]. Since its use has been primarily in a mouse model, this linker gene was re-designed, 5'-AAC GAC GCC CAG GCC CCC AAG AGC/TCA-3', to optimize the codon bias in favor of expression in bovine cells (http://www.kazusa.or.jp/codon/) [395].

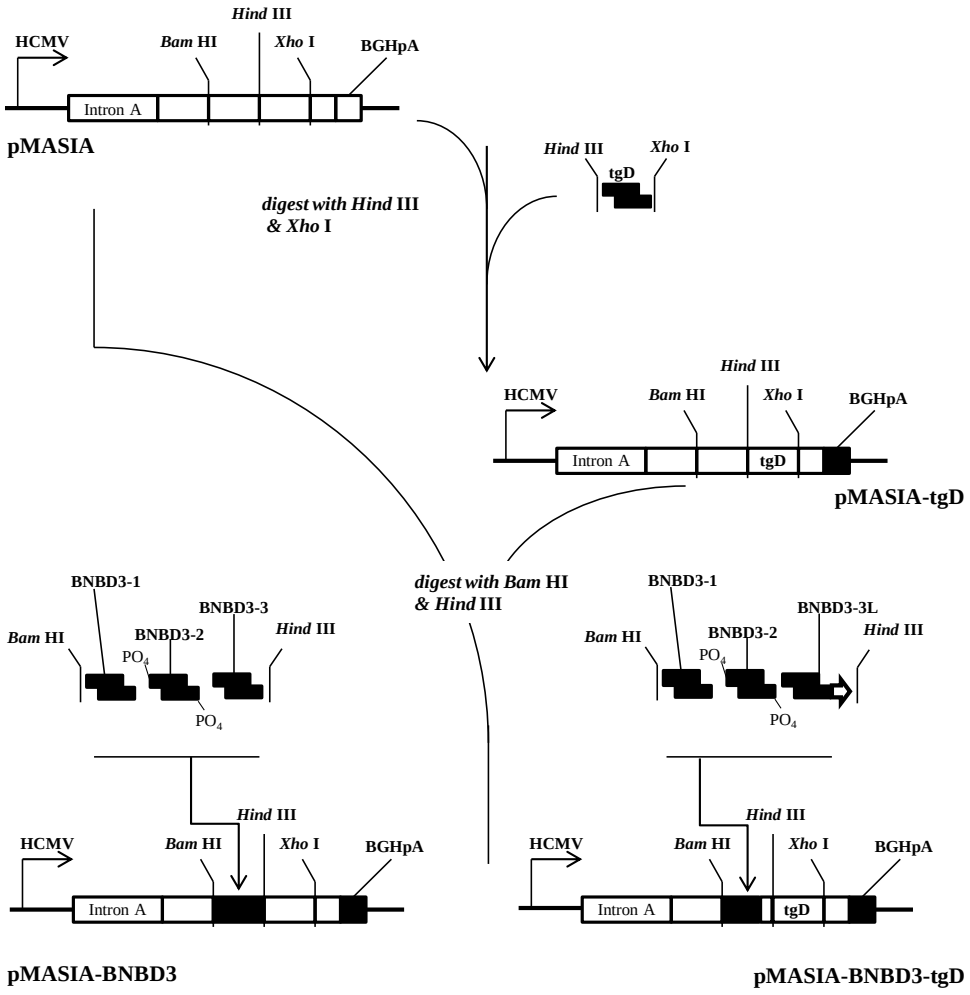

Figure 4.1 Schematic diagram of the construction of pMASIA-tgD, pMASIA-BNBD3 and pMASIA-BNBD3-tgD. The gene encoding BoHV-1 tgD was cloned into pMASIA to create pMASIA-tgD. To construct pMASIA-BNBD3, complimentary oligonucleotides encoding the mature sequence of BNBD3 were synthesized as 3 sticky-ended pieces (BNBD-1, BNBD-2, BNBD-3) and inserted into pMASIA using the BamHI and HindIII sites. For construction of pMASIA-BNBD3-tgD, BNBD3 was similarly inserted in 3 pieces into pMASIA-tgD, and only the 3rd piece (BNBD3-3L) differed with a linker sequence at the 3' end.

Synthetic oligonucleotides were suspended, and the complimentary pairs were annealed according to the supplier's instructions. Then the three double-stranded sticky-ended oligonucleotide pieces encoding BNBD3 were ligated into the BamHI/HindIII site of pMASIA and pMASIA-tgD, respectively. The ligation reactions were used to transform *Escherichia coli* strain JM109 and after selection (Kanamycin+) and amplification, the plasmids were purified using a QIAprep Spin Miniprep kit (Qiagen). Correctness of pMASIA-BNBD3 and pMASIA-BNBD3-tgD was verified by restriction digestion and confirmed by DNA sequencing. Plasmids were amplified in *Escherichia coli* JM109 cells and purified with Endofree Plasmid Giga kits (Qiagen).

Table 4.1 BNBD3-encoding complimentary synthetic oligonucleotide pairs

Name	Primer or Oligonucleotide sequence
BNBD3-1	5'-GATCCAT**ATG**CAAGGAGTAAGAAATCATGTAACCTGCCGTATAAATAGAGGCT-3' [a]
BNBD3-1c	5'-CAGAAGCCTCTATTTATACGGCAGGTTACATGATTTCTTACTCCTTG**CAT**ATG-3' [a]
BNBD3-2	5'-*TCTG*TGTGCCGATCAGGTGCCCTGGACGCACGAGACAGATTGGCACCTGTTTC-3' [b]
BNBD3-2c	5'-*GCCC*GAAACAGGTGCCAATCTGTCTCGTGCGTCCAGGGCACCTGATCGGCACA-3' [b]
BNBD3nL-3	5'-GGGCCCCGAATAAAATGCTGCAGGTCGTGG**TAG**A-3' [c]
BNBD3nL-3c	5'-AGCTT**CTA**CCACGACCTGCAGCATTTTATTCGGG-3' [c]
BNBD3L-3	5'-GGGCCCCGAATAAAATGCTGCAGGTCGTGG*AACGACGCCCAGGCCCCCAAGAGC*A-3' [d]
BNBD3L-3c	5'-AGCTT*GCTCTTGGGGGCCTGGGCGTCGTT*CCACGACCTGCAGCATTTTATTCGGG-3' [d]

[a] First piece of BNBD3; a 5' BamHI restriction site is underlined, and the start codon is in bold.
[b] Second piece of BNBD3; oligonucleotides were 5' phosphorylated, and sticky ends were made by a 4-nucleotide overhang (italics).
[c] Third piece of BNBD3 (pMASIA-BNBD3); a 3' HindIII restriction site is underlined, and the TAG stop codon is in bold.
[d] Third piece of BNBD3 (pMASIA-BNBD3-tgD); the bovine codon optomised linker is in italic, and a 3' HindIII restriction site is underlined.

4.3.2 Expression of BNBD3 and BNBD3-tgD in vitro

COS7 cells at 50-80% confluency were transiently transfected with pMASIA-BNBD3 or pMASIA-BNBD3-tgD using Lipofectamine Plus Reagent in Optimem (Life Technologies). After 48 h the cell supernatants were collected, clarified by centrifugation and concentrated 10X using an Amicon Ultra 10kDa (pMASIA-tgD, pMASIA-BNBD3-tgD) or Amicon Ultra 3kDa (pMASIA-BNBD3) centrifugal filter (Millipore, Bedford, MA, USA). Supernatants were divided to provide samples for Coomassie blue staining and Western blotting, and prepared in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer in the presence or absence of 2-mercaptoethanol.

Proteins were separated by SDS-PAGE on a 10% acrylamide gel (pMASIA-tgD, pMASIA-BNBD3-tgD) according to the method of Laemmli [396] or by tricine SDS-PAGE on a 15% acrylamide gel (pMASIA-BNBD3) according to the method of Shagger [397, 398]. Proteins were transferred onto 0.45 μm nitrocellulose (pMASIA-tgD, pMASIA-BNBD3-tgD) or 0.2 μm PVDF (pMASIA-BNBD3) membranes. The membranes were washed in TBST (0.15M Tris, 0.02M NaCL [pH 7.5], 0.1% Tween 20) and then incubated for 2 h at RT or overnight at 4°C in TBST containing 3% skim milk powder (SMP). Membranes were probed with gD-specific monoclonal antibody (mAb) 3D9S diluted 1:4000 in TBST/1% SMP [399], or with BNBD3-specific rabbit serum diluted 1:2000 in TBST/1% SMP for 2 h at RT. Polyclonal antibodies to BNBD3 were generated as described previously [400]. The synthetic peptide, QGVRNHVTCRINRGFCVPIRCPGRTRQIGTCFGPRIKCCRSW, which corresponds to the published amino-acid sequence for the mature form of BNBD3 (UniProtKB/Swiss-Prot

accession number P46161) [201, 219, 389] was used for immunization. The membranes were washed in TBST and then incubated with alkaline phosphatase (AP)-conjugated mouse- or rabbit-specific IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) at a dilution of 1/5000 in TBST/1% SMP for 1 h at RT. The bound antibodies were visualized with 5-bromo-4-chloro-3-indolylphosphate (BICP)/ nitroblue tetrazolium (NBT) substrate (Sigma). Images of the Western blots were captured and processed using a GelDoc XR imaging system (Bio-Rad Laboratories Ltd, Randolph, MA, USA).

4.3.3 Immunizations and BoHV-1 challenge

Six to eight week-old C56BL/6 mice (8 mice per group) were immunized twice at a 4-week interval intradermally (ID) at the base of the tail with 5 µg pMASIA, pMASIA-tgD, pMASIA-BNBD3-tgD, or a mixture of pMASIA and pMASIA-tgD or pMASIA-BNBD3 and pMASIA-tgD. One month after the final vaccination, mice were euthanized. Serum was taken to be assayed for tgD-specific antibody levels and spleens were collected for analysis of tgD-specific CMI responses.

Eight to nine month-old BoHV-1 seronegative Angus and Hereford crossbred calves were randomly allocated to five groups of six animals each and immunized with 750 µg pMASIA, pMASIA-tgD, pMASIA-BNBD3-tgD, a mixture of pMASIA and pMASIA-tgD, or a mixture of pMASIA-BNBD3 and pMASIA-tgD. The plasmids were delivered three times at four-week intervals ID in the neck with a needle-free delivery device (Biojector Medical Technologies, Portland, OR). One month after each vaccination, serum was collected for analysis of tgD-specific antibody levels and peripheral blood was collected for analysis of tgD-specific CMI

responses.

Calves in the groups immunized with pMASIA, pMASIA-tgD or pMASIA-BNBD3-tgD were challenged intranasally with 4 ml of aerosolized 10^7 pfu/ml BoHV-1 strain 108, 52 days after the last immunization. Sera were collected prior to, and on days 8 and 16 after challenge. Peripheral blood was collected and CMI responses were measured prior to challenge and on day 8 after challenge by proliferation and ELISPOT assays; on day 16 by ELISPOT depletion assay; and on day 25 by a 4-color CTL assay. Calves were clinically assessed before challenge and for 10 days after challenge. Temperatures and body weights were measured and nasal swabs were collected every second day. All procedures were approved by the University Council for Animal Care and Supply in accordance with the standards stipulated by the Canadian Council on Animal Care.

4.3.4 Serology

For the enzyme-linked-immunosorbent assay (ELISA), ninety-six-well polystyrene microtiter plates (IMMULON® 2; Thermo Electron Corrp., Milford, MA) were coated overnight at 4°C with 0.05 µg of tgD per well in sodium carbonate coating buffer. Plates were washed in PBS with 0.05% Tween 20 (PBST) and then incubated overnight at 4°C with serially diluted mouse sera starting at 1:40 in four-fold dilution, with all dilutions in PBS containing 0.5% gelatin (PBS-g). Plates were washed and bound IgG was detected using AP-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories) diluted 1:5000 in PBS-g for 1 h at room temperature (RT). Bovine sera were diluted four-fold in PBS-g starting at 1:10, added to plates and incubated for 2 h at RT. The plates were then incubated for 1 h at RT with affinity-purified AP-conjugated goat anti-bovine IgG (Kirkegaard & Perry Laboratories) diluted 1:10,000 in PBS-g. All reactions

were visualized with 0.01M *p*-nitrophenyl phosphate (PNPP) (Sigma-Aldrich) in 0.104 M diethanolamine, 0.5 mM MgCl. Absorbance was read on a model 3550 Microplate Reader (Bio-Rad Laboratories Ltd) at 405 nm, with a reference wavelength of 490 nm. ELISA titers were expressed as the inverse of the serum dilution that gave an absorbance (*A*) value two standard deviations above the values for serum from control naïve animals.

Virus neutralization (VN) titers in cattle sera were determined as described previously [401]. Viral plaques were visualized by staining each well with 20 µl of 0.5% crystal violet in 80% methanol for 1 min., and then counted. Titers were expressed as the reciprocal of the highest dilution of serum that resulted in a 50% reduction in plaques relative to the serum-free positive virus control [44].

4.3.5 Enzyme-linked-immunospot assay (ELISPOT)

Splenocytes were isolated and resuspended as previously described [402] at a concentration of 1 x 10^7 cells per ml in complete medium (cRPMI) made up of RPMI 1640, supplemented with 10% fetal bovine serum (FBS), 50 µg/ml gentamycin, 1 mM L-glutamine, 10 mM Hepes, 1 mM non-essential amino acids, 1 mM sodium pyruvate (all Life Technologies Inc., Burlington, ON, Canada) and 5 x 10^{-5} M 2-mercaptoethanol (Sigma-Aldrich). Nitrocellulose plates (96-well Multiscreen-HA; Millipore Corp., Bedford, MA, USA) were coated overnight at 4°C with 0.2 µg/well of anti-mouse IFN-γ or IL-5 (BD Biosciences, San Jose, CA, USA). Plates were washed with PBS and then blocked with 1% (w/v) bovine serum albumin (BSA; Sigma-Aldrich) for 2 h at 37°C. Splenocytes at 10^6 cells/well were added to triplicate wells containing medium or tgD at 3 µg/ml and incubated at 37°C for 20 h. Plates were washed and then incubated with 2 µg/ml biotinylated rat anti-mouse IFN-γ or IL-5 (BD Biosciences) in PBS with 1% BSA for 1.5 h,

followed by streptavidin-AP (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted 1:1000 in PBS with 1% BSA for 1.5 h.

For cattle, the ELISPOT assay was performed as described above for mice, but with the following changes. Blood was collected into EDTA, and peripheral blood mononuclear cells (PBMCs) were isolated on Ficoll-Paque Plus (Pharmacia, Mississauga, ON, Canada) as previously described [263] and suspended at 1 x 10^7 cells/ml in cRPMI. To assess the IFN-γ response attributable to the CD8 and/or CD4 subsets, the ELISPOT assay was also conducted with PBMCs that were first depleted of either the CD8 or CD4 T cells using magnetic-activated cell sorting (MACs) and subsequent flow cytometry to verify cell purity as has been previously described in detail [403]. Positively selected CD4 cells or CD8 cells were added back to their respective depleted populations as a percentage of 10^6 cells per well. For example, in the $CD4^-$ + 5% CD4+ sample, where the number of $CD4^-$ cells was 0.95 x 10^6 cells/well (95%), 50,000 (5%) $CD4^+$ cells were added to the well to give a total of 10^6 cells/well. Plates were coated with a bovine IFN-γ-specific mAb (2-2-1) [404]. IFNγ-secreting cells were identified with 1:1500 rabbit serum specific for bovine IFN-γ (lot 92-131) [404] followed by AP-conjugated goat anti-rabbit IgG (Kirkegaard & Perry Laboratories).

Spots representing IFN-γ or IL-5 secreting splenocytes in mice or IFN-γ secreting PBMCs in cattle were visualized using BCIP/NBT substrate (Sigma-Aldrich). Stained spots were counted and the number of IFN-γ or IL-5 secreting cells per 1 x 10^6 cells was expressed as the difference between the number of spots in the tgD-stimulated wells and the number of spots in the control (medium) wells.

4.3.6 Proliferation:[methyl-^{3}H] thymidine incorporation assay

Bovine blood was collected, and PBMCs were isolated and suspended as described above. Cells were further diluted to a concentration of 3.5 x 10^6 cells/ml in cRPMI. One hundred µl of this suspension was dispensed in triplicate into wells containing 100 µl of either medium or tgD at 3 µg/ml, and then plates were incubated at 37°C. Following 72 h of restimulation, the cells were pulsed with 0.4 µCi/well of [methyl-^{3}H] thymidine (Amersham Biosciences, Baie d'Urfe, PQ, Canada). After an additional 18 h of culture, cells were collected with a Filtermate harvester and thymidine uptake was measured by scintillation counting with a TopCount NXT microplate scintillation counter (Packard Instrument Company, Meriden, CT, USA). Proliferative responses were calculated as the means of triplicate wells and expressed as a stimulation index (SI) (counts per minute in the presence of antigen/counts per minute in the absence of antigen).

4.3.7 Antigen-specific CD8$^+$/IFNγ$^+$ cytotoxic T cell assay

In mice the induction of CTLs, defined as CD8$^+$ CD3$^+$ T-cells that when stimulated by recall antigen secrete IFN-γ [405-408], was measured by fluorescence-activated cell sorting (FACS). Splenocytes were isolated from each mouse as described above. Pooled splenocytes [408, 409] from each vaccination group were added to wells of a 96-well plate at 1 x 10^6 cells/well, and then cultured at 37°C in the absence or presence of tgD at 3 µg/ml for 7 h. GolgiPlug (BD Biosciences) was added 5 h before harvesting the cells. Cells were washed once with FACS buffer (PBS pH 7.2, 0.1% BSA, 0.05% $NaNH_3$) supplemented with 2% FBS and cell-surface stained with PE anti-mouse CD3 (IgG2b; clone 17A2, BD Biosciences) and FITC anti-mouse CD8 (IgG2a; clone 53-6.7, BD Biosciences) at 4°C for 30 min. For intracellular cytokine staining of IFN-γ, cells were washed, fixed and permeablized using the Cytofix/Cytoperm kit

according to the manufacturer's instruction (BD Biosciences). Intracellular IFN-γ was stained with APC anti-mouse IFN-γ (IgG1; clone XMG1.2, BD Biosciences).

In cattle, due to difficulty doing CTL assays in outbred animals, the induction of activated $CD8^+$ IFN-γ^+ cells in the PBMCs was used as a measurement of CTL response using a FACs-based assay. This assay has been accepted as an alternative in other species to the standard cytolytic (Cr_{51} release) assay [410, 411], and was also selected based on a published report of CTL identification in cattle as activated, $CD8^+$, IFNγ-expressing cells [313]. Accordingly, $CD8^+$ and IFN-γ^+ expression, concurrent with CD25 (activation), were used as surrogate markers for CTLs [406, 412], and thus, cells were identified using a modification of a method that allows for simultaneous detection of expression of $CD8^+$ and IFN-γ^+ with the cell activation marker CD25 [413-416]. Optimal conditions for cell collection and culture were determined in preliminary studies. Blood from two animals from the pMASIA-tgD, pMASIA-BNBD3-tgD and pMASIA groups was collected into heparin. PBMCs were isolated on Ficoll-Paque Plus (Pharmacia) [389], suspended in cRPMI and then added at 1 x 10^6 cells per well in 250 µl volume of a 96-well round-bottom tissue-culture microtitre plate (Corning Costar; Thermo Fisher Scientific, Life Technologies Inc., Burlington, ON, Canada). The cells were cultured at 37°C in the absence or presence of tgD at 1 µg/ml for 4 days. GolgiStop (BD Biosciences) was added for 12 h before harvesting the cells. Cells were washed twice with, and resuspended in, 50 µl of FACS buffer and surface-stained using an indirect staining method. Cells were incubated singly with mouse anti-bovine CD3 mAb (IgG1, MM1A, VMRD, Pullman, WA, USA), and singly or with a mAb mix that consisted of i) mouse anti-bovine CD8 (IgG1, CACT80C, VMRD), mouse anti-bovine γδTCR (gamma-delta T cell receptor) (IgG2b, GB21A, VMRD) and mouse anti-bovine CD25

(IgG2a, CACT108A, VMRD), or ii) mouse anti-bovine CD4 (IgG1, CACT138A, VMRD), mouse anti-bovine γδTCR and mouse anti-bovine CD25, for 30 min at 4°C. After washing twice with FACs buffer, cells were incubated for 30 min at 4°C either singly or with a mixture of the appropriate secondary reagent(s) goat $F(ab')_2$ anti-mouse IgG1-FITC (1072-02, Southern Biotech, Birmingham, AL, USA), goat anti-mouse IgG2b-TRI-COLOR® (M32406, Molecular Probes, Life Technologies Inc.), and goat $F(ab')_2$ anti-mouse IgG2a-RPE (1082-09, Southern Biotech). Intracellular cytokine staining of IFN-γ was accomplished with a two-step indirect method using the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions with the following exceptions. Cells were fixed and permeabilized by incubating at 4°C for 1.5 h. Cells were washed twice and resuspended in 1xBD Perm/Wash for both steps. In the first step, 25 µl of prediluted biotinylated mouse anti-bovine IFN-γ (biotin MCA1783B, AbD Serotec) was added for 1 h at 4°C. In the second step, 25 µl of prediluted streptavidin-AlexaFluor® 647 conjugate (streptavidin S-32357, Molecular Probes, Invitrogen) was added for 30 min at 4°C.

Staining specificity was controlled with the appropriate isotype-matched antibody controls. Samples were resuspended in Flow buffer (PBS, 1% ultrapure formaldehyde) and kept in the dark at 4°C until flow cytometric acquisition was performed. Single-stained samples were used to set compensation levels for acquisition of multi-color stained samples. Specific CTL responses, evaluated as the increase in the percentage of $CD3^+CD8^+IFN\gamma^+$ cells in mice and $CD25^+CD8^+IFN\gamma^+$ cells in cattle [313, 406, 409, 412, 417] in response to *in vitro* tgD stimulation were detected by flow cytometric analysis. Samples were acquired using a FACSCalibur flow cytometer and the data were analyzed with CELLQuest software (BD Biosciences).

4.3.8 Statistical Analysis

All data were analysed with the aid of GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). For mouse and cattle pre-challenge ELISA titers and ELISPOT counts, cattle pre-challenge proliferation assay SIs and cattle post-challenge ELISPOT counts, differences among groups were examined using the non-parametric Kruskal-Wallis test. If the result of an analysis of variance (ANOVA) proved significant, then multiple post-test comparisons between medians were done using a Dunn's test, or differences between the medians of two groups were examined using the Mann-Whitney U test. Post-challenge cattle ELISA and virus neutralisation titers, virus shedding, weight change and temperature differences among groups over time were analysed by a two-way ANOVA followed by a Bonferroni *t*-test in case of a significant ANOVA. Differences between groups were considered significant if probability values of $p<0.05$ were obtained.

4.4 Results

4.4.1 In vitro expression of BNBD3 and BNBD3-tgD in COS-7 cells

Expression of BNBD3 from pMASIA-BNBD3 was verified by Western blot analysis. A single band of the expected molecular mass (4.8kDa) [219] that co-migrated with the synthesized BNBD3 control (Figure 4.2a,c; lanes 2,3), was detected in the supernatants from pMASIA-BNBD3 transfected cells (Figure 4.2a,c; lane 5) under both reducing (Figure 4.2a) and non-reducing (Figure 4.2c) conditions. However, BNBD3 was not detected in the supernatants from pMASIA-transfected cells (Figure 4.2a,c; lane 4). A protein corresponding to the expected molecular mass of tgD (61kDa), was revealed by gD-specific mAb in supernatants from transfections with pMASIA-tgD (Figure 4.2b,d; lane 3, right panel) or pMASIA-BNBD-tgD

(Figure 4.2b,d; lane 4, right panel), under both reducing (Figure 4.2b) and non-reducing (Figure 4.2d) conditions, thus verifying expression of tgD from both plasmids. The BNBD3-specific rabbit serum reacted only with the supernatant from pMASIA-BNBD3-tgD transfection (Figure 4.2b,d; lane 4; left panel) but not with the supernatant from pMASIA-tgD transfection (Figure 4.2b,d; lane 3; left panel) under both reducing (Figure 4.2b) and non-reducing (Figure 4.2d) conditions.

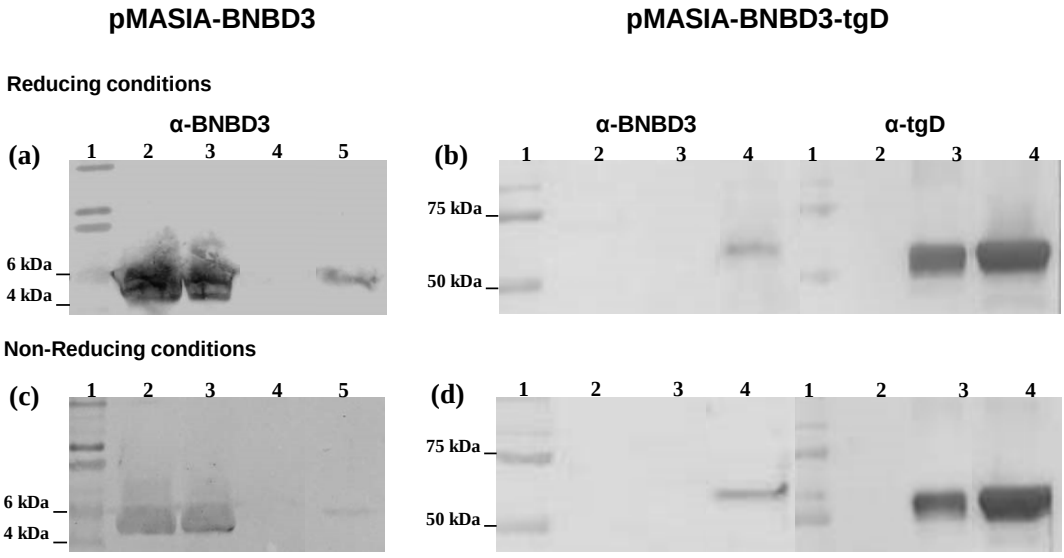

Figure 4.2 *In-vitro* expression of the BNBD3-encoding constructs. (a-d) Cos-7 cells were transiently transfected with (a,c) pMASIA-BNBD3 or (b,d) pMASIA -BNBD3-tgD. The presence of BNBD3 and tgD in the supernatants of transfected cells was detected at 48 h post-transfection by Western blotting with rabbit anti-BNBD3 polyclonal or anti-gD monoclonal antibodies under (a,b) reducing and (c,d) non-reducing conditions. (a,c) 1- molecular weight marker, 2-sBNBD3 peptide (1 µg), 3- sBND3 peptide (0.5 µg), 4- pMASIA supernatant, 5-pMASIA-BNBD3 supernatant (b,d) 1- molecular weight marker (kDa), 2- pMASIA supernatant, 3- pMASIA-tgD supernatant , 4- pMASIA-BNBD3-tgD supernatant. (sBNBD3 = synthesized BNBD3).

As expected, there was little difference in the molecular weight of the fusion protein BNBD3-tgD, which is approximated as 65.4 kDa, versus that of tgD (61 kDa) due to the small molecular mass of BNBD3 (4.8 kDa). These results verified the expression of BNBD3 and BNBD3-tgD, both in monomeric form, from eukaryotic cells transfected with pMASIA-BNBD3 and pMASIA-BNBD3-tgD, respectively.

4.4.2 Immune responses induced by BNBD3-encoding DNA vaccines in mice

The capacity of BNBD3, encoded either on a separate plasmid or as a fusion construct with tgD, to enhance tgD-specific immune responses was first evaluated in mice. Mice immunized with pMASIA + pMASIA-tgD or pMASIA-BNBD3 + pMASIA-tgD developed significantly higher IgG titers when compared to the animals immunized with

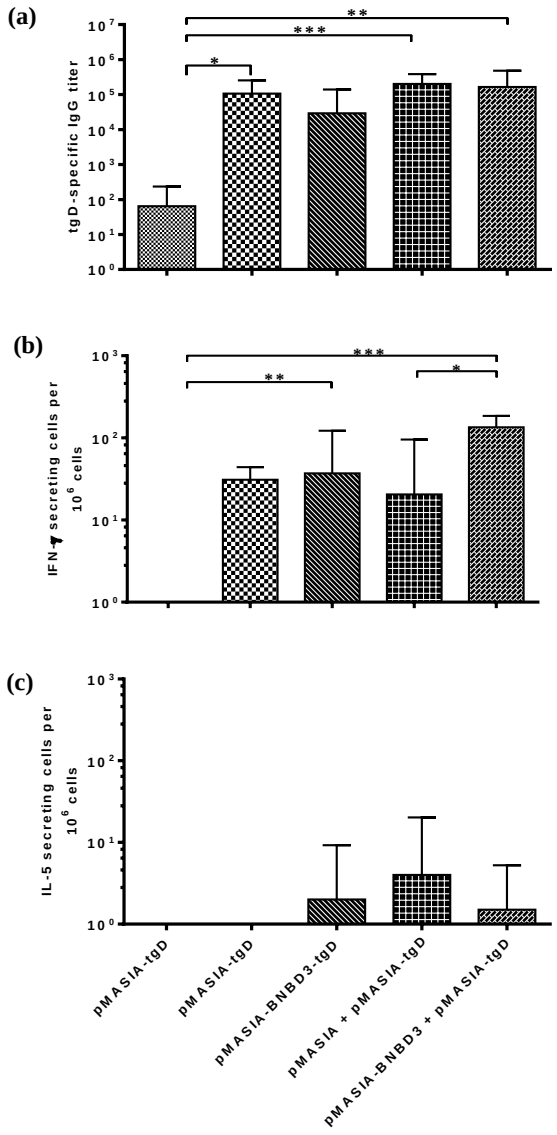

Figure 4.3 tgD-specific immune responses in mice immunized with plasmids encoding tgD and/or BNBD3. C57BL/6 mice (8 mice per group) were immunized twice ID with 5 µg plasmid. One month after the second immunization, tgD-specific IgG titers were determined by ELISA (a). ELISA titers are expressed as the reciprocal of the highest dilution resulting in a reading of two standard deviations above the negative control. One month after the second immunization, the numbers of tgD-specific (b) IFN-γ or (c) IL-5 secreting cells were measured by ELISPOT assay. ELISPOT results are expressed as the difference between the number of IFN-γ or IL5 secreting cells in tgD-stimulated wells and medium-control wells per 10^6 cells. Bars represent the median values of each group with interquartile range. Significant differences between groups are indicated on the graphs where *p<0.05, **p<0.01 and ***p<0.001. Similar results were obtained in a second independent experiment.

pMASIA ($P < 0.01$), but there was no difference between these groups (Figure 4.3a). The IgG titer of the group immunized with pMASIA-BNBD3-tgD appeared to be lower, but was not significantly different when compared to that of the pMASIA-tgD group. However, only mice immunized with pMASIA-tgD and not pMASIA-BNBD3-tgD, developed significantly higher IgG titers than the group immunized with pMASIA ($P<0.05$) (Figure 4.3a). These results demonstrate that despite these trends, BNBD3 had no significant effects on the humoral immune response induced by tgD.

To examine the effects of BNBD3 on the CMI responses, tgD-induced secretion of IFN-γ and IL-5 by splenocytes was measured by ELISPOT assay. Mice immunized with pMASIA-BNBD3 + pMASIA-tgD had a significantly higher number ($p<0.001$) of IFN-γ-secreting cells upon restimulation with tgD when compared to the animals immunized with pMASIA and when compared to their control group immunized with pMASIA + pMASIA-tgD ($p<0.05$) (Figure 4.3b). Immunization with pMASIA-BNBD3-tgD increased the number of IFN-γ-secreting cells ($p<0.01$) when compared with pMASIA, but there was no significant difference with pMASIA-tgD. The number of cells expressing IL-5 was very low in all vaccinated groups (Figure 4.3c). These results indicate that addition of BNBD3 by separate plasmid resulted in increased numbers of IFN-γ secreting splenocytes, while BNBD3 added to tgD as a fusion had no effect on the IFN-γ response in mice.

In earlier DNA vaccine studies involving mBD2 in the mouse model it was found that physical linkage between the β-defensin and antigen was required for improvement of immune responses [217, 218, 391], and that this may have been particularly important for development of the CTL

response [218]. To investigate the influence of BNBD3 as a fusion with tgD on the induction of $CD8^+IFN\text{-}\gamma^+$ CTLs, the tgD-restimulated splenocytes from the mice immunized with pMASIA-BNBD3-tgD were evaluated by FACs analysis for surface expression of CD3, CD8 and concurrent intracellular expression of IFN-γ. First, the live (single) cells were gated according to forward scattered (FSC) and side scattered (SSC) light properties (Figure 4.4a) [418]. Analysis of the total live cells showed that vaccination with pMASIA-BNBD3-tgD induced a greater percentage of tgD-specific $CD8^+$ IFN-γ^+ cells (8.7%. Figure 4.4c,d) than was induced in the group vaccinated with pMASIA-tgD (0.1%. Figure 4.4b,d) or vaccinated with pMASIA (0%, Figure 4.4d). Next, the live cells were further gated on surface expression of CD3 to exclude all non-T cells (Figure 4.4e). Analysis of this $CD3^+$ population showed that vaccination with pMASIA-BNBD3-tgD again induced a greater percentage of tgD-specific $CD8^+$ IFN-γ^+ cells (16.1%. Figure 4.4g,h) than was induced by vaccination with pMASIA-tgD (11.7%, Figure 4.4F,H) or with pMASIA (0%, Figure 4.4h), but the effect of BNBD3 was not as pronounced in this $CD3^+$ T cell only population. When the frequencies of $CD8^+$ IFN-γ^+ cells in the total live cell population (Figure 4.4d) and in the $CD3^+$ cell populations (Figure 4.4h) were compared, it appeared that vaccination with pMASIA-BNBD3-tgD induced a population of non-T cell ($CD3^-$) IFN-γ^+-secreting $CD8^+$ cells that vaccination with pMASIA-tgD did not. Since DCs are currently the only known non-T $CD8\alpha^+$ cells in the mouse spleen, it is possible that the $CD3^-$ $CD8^+$ IFN-γ^+ cells that we observed were splenic DC [419]. These results demonstrate that addition of BNBD3 as a fusion construct led to enhanced induction of tgD-specific CTL, and thus a Th1-type, response.

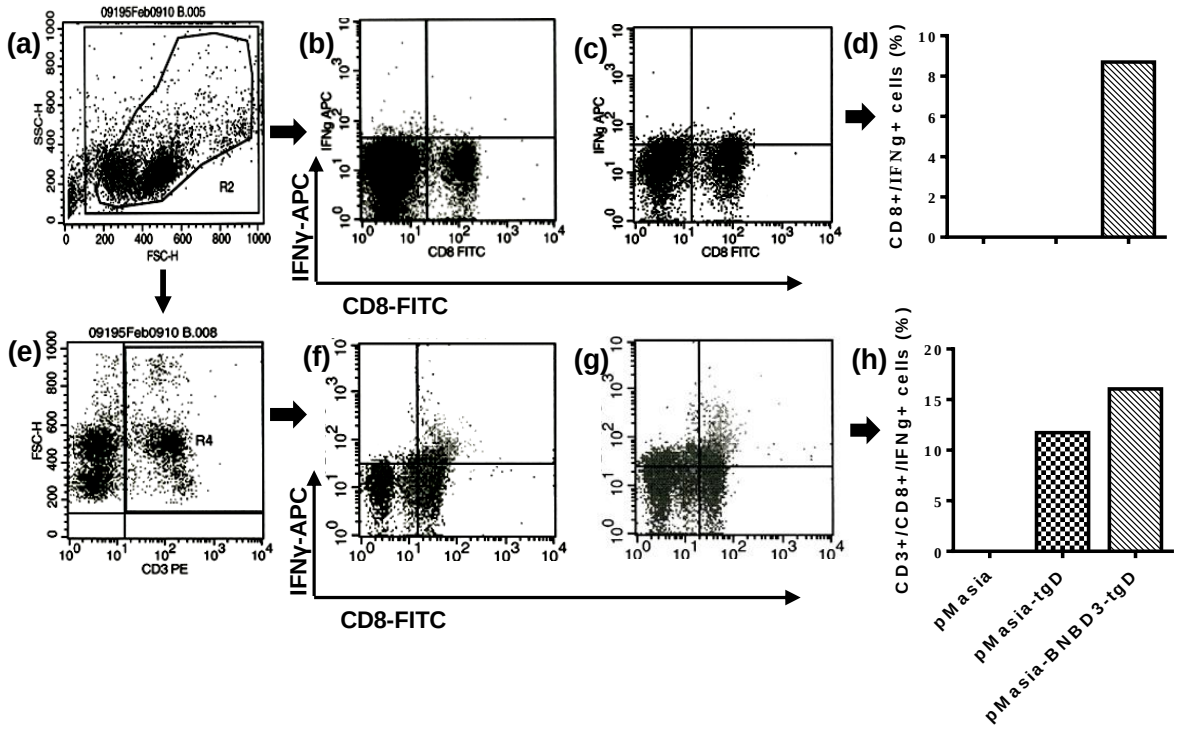

Figure 4.4 IFN-γ/CD8+ cells in mice immunized with plasmids encoding tgD with or withouot BNBD3. C57BL/6 mice (8 mice per group) were immunized twice ID with 5 µg plasmid. Splenocytes from immunized animals from each group were pooled and duplicate wells were restimulated *in vitro* without or with tgD for 7 h (in the presence of Brefeldin A for the last 5 h). Cells were triple-stained for surface expression of CD8 and CD3 and for intracellular expression of IFN-γ. Specific $CD8^+$/ IFN-γ^+ cells were detected by flow cytometry. (a,e) Representative dot plots show the gating strategy used to (b,c,f,g) analyze IFN-γ expression. (a) First, dead cells and doublets were excluded and live cells were gated in a forward scatter-height (FSC-H) against side scatter-height (SSC-H) dot plot. (b) $CD8^+$/IFN-γ^+ cells in the live cell gate include non-T cells from mice vaccinated with pMASIA-tgD or (c) pMASIA-BNBD3-tgD. (d) Data for $CD8^+$/ IFN-γ^+ cells as a % of total live cells are shown in graph form. (e) T cells were selected from total live cells by gating on $CD3^+$ events. (f) $CD8^+$/IFN-γ^+ cells in the $CD3^+$ gate include only T cells from mice vaccinated with pMASIA-tgD or (g) pMASIA-BNBD3-tgD. (h) Data for $CD8^+$/IFN-γ^+ cells in the CD3 gate are shown in graph form. Results are expressed as the difference between the % of IFN-γ secreting cells in tgD-stimulated wells and non-stimulated medium-control wells. Bars represent the mean value of the duplicate wells of pooled cells from the 8 mice in each treatment group. Similar results were obtained in a second independent experiment.

4.4.3 Immune responses induced by BNBD3-encoding DNA vaccines in cattle

Once it was confirmed that plasmids encoding BNBD3 induced immune responses in mice, we next assessed the effects of these plasmids in cattle. Calves immunized with either pMASIA-BNBD3-tgD or pMASIA-BNBD3 + pMASIA-tgD did not develop higher IgG titers than animals immunized with pMASIA, whereas calves immunized with pMASIA-tgD or pMASIA + pMASIA-tgD ($p<0.01$) did have a significantly higher IgG titer (Figure 4.5a). These results demonstrate that addition of BNBD3, encoded either on a separate plasmid or as a fusion construct with tgD, did not enhance the humoral immune response induced by pMASIA-tgD in cattle.

Proliferative responses of tgD-restimulated PBMCs from calves immunized with pMASIA-BNBD3 + pMASIA-tgD were not significantly improved when compared to its control group pMASIA + pMASIA-tgD, and most importantly, neither group was able to develop significantly higher proliferation when compared to calves in the group immunized with pMASIA (Figure 4.5b).In contrast, addition of BNBD3 to tgD as a fusion construct greatly improved the proliferative response when compared to pMASIA-tgD ($P<0.01$).Notably, the group immunized with pMASIA-BNBD3-tgD was the only one with significantly higher proliferation than the group immunized with pMASIA (Figure 4.5b). To further confirm activation and to characterize the type of immune response generated, production of IFN-γ was assessed (Figure 4.5c). Neither of the two-plasmid immunization strategies, pMASIA + pMASIA-tgD or pMASIA-BNBD3 + pMASIA-tgD, induced a significantly greater number of IFN-γ –secreting cells when compared to pMASIA. In contrast, addition of

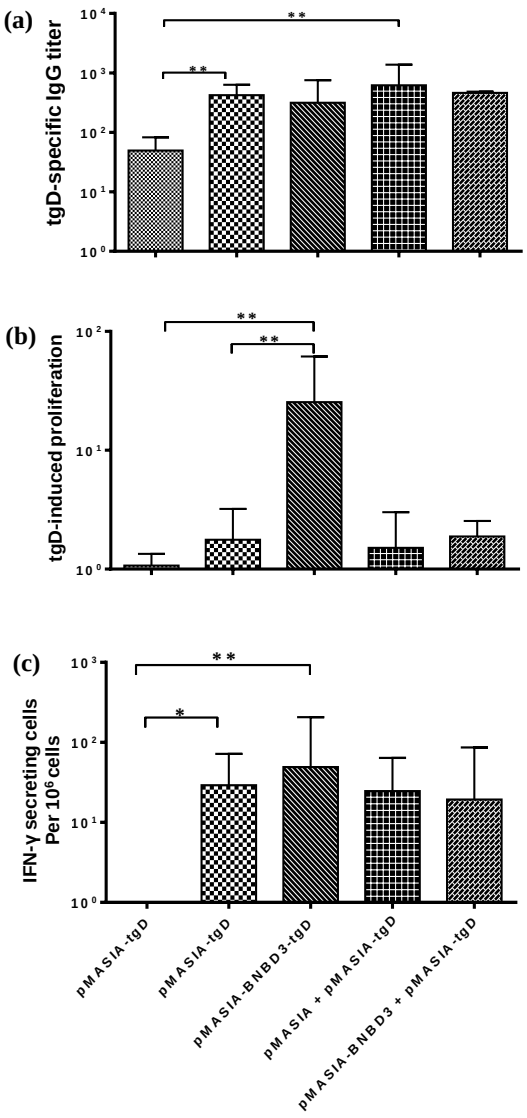

Figure 4.5 tgD-specific immune responses of calves immunized with plasmids encoding tgD and/ or BNBD3. Eight to nine month-old BoHV-1 seronegative Angus and Hereford crossbred calves were immunized three times ID by needle-free injection with 0.75 mg plasmid and responses were determined one month after the third immunization (6 animals per group). (a) Serum tgD-specific IgG was measured by ELISA and the titers are expressed as the reciprocal of the highest dilution resulting in a reading of two standard deviations above the negative control. (b) tgD-specific lymphocyte proliferation of PBMCs cultured in medium or with 3 µg/ml of tgD and pulsed with 0.4 µCi/well of methyl-^{3}H] thymidine. Results are expressed as a SI. (c) The number of IFN-γ-secreting cells measured by ELISPOT assay. ELISPOT results are expressed as the difference between the number of IFN-γ secreting cells in tgD-stimulated wells and medium-control wells per 10^6 cells. Bars represent the median values of each group with interquartile range. Significant differences between groups are indicated on the graphs where *p<0.05 and **p<0.01.

BNBD3 to tgD as a fusion construct resulted in an increase in the number of IFN-γ secreting cells. Although this increase was not statistically significant when compared to its control group pMASIA-tgD, the significance of the difference observed between pMASIA and pMASIA-BNBD3-tgD ($p<0.01$) was greater than that of the difference between pMASIA and pMASIA-tgD ($p<0.5$). Thus, in cattle the addition of BNBD3 as a fusion construct, but not when delivered by separate plasmid, enhanced the proliferative response to pMASIA-tgD.

4.4.4 Immune responses of cattle after immunization with DNA vaccines and BoHV-1 challenge

Since BNBD3, when delivered on a separate plasmid, did not improve immune responses to tgD, the pMASIA-BNBD3 + pMASIA-tgD and the pMASIA + pMASIA-tgD groups were not further evaluated, while the groups immunized with pMASIA, pMASIA-tgD or pMASIA-BNBD3-tgD were subsequently challenged with BoHV-1. Throughout the period after challenge, calves immunized with pMASIA-tgD ($p<0.01$) had higher serum tgD-specific IgG titers than calves immunized with pMASIA (Figure 4.6a), while the VN titers were significantly higher in both pMASIA-tgD and pMASIA-tgD-BNBD3 immunized groups when compared to the pMASIA group (Figure 4.6b). With exception of the IgG titers on day 16-post challenge, no differences were observed between the pMASIA-tgD and pMASIA-tgD-BNBD3 groups. In contrast, addition of BNBD3 to tgD as a fusion construct resulted in a significant increase in the number of IFN-γ secreting cells ($p<0.05$) when compared to the pMASIA-tgD group (Figure 4.6c).

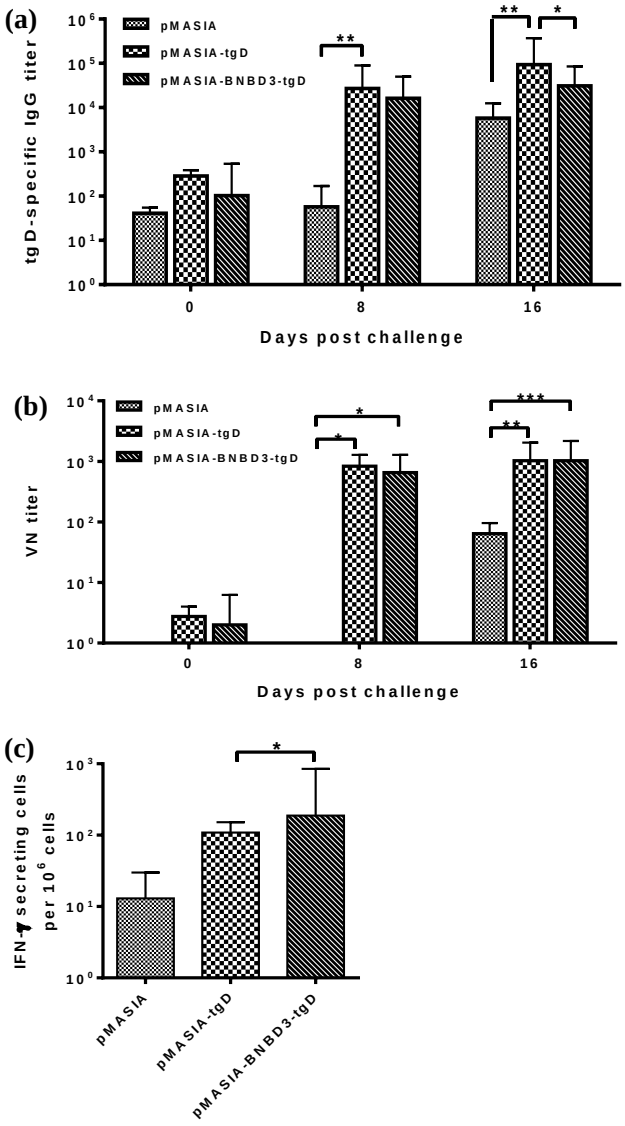

Figure 4.6 Immune responses of calves immunized with plasmids encoding tgD and/or BNBD3, and challenged with BoHV-1. Eight to nine month-old BoHV-1 seronegative Angus and Hereford crossbred calves were immunized three times ID by needle-free injection with 0.75 mg plasmid followed by BoHV-1 challenge 52 days after the third immunization (6 animals per group). (a) Serum tgD-specific IgG was measured by ELISA on the day of challenge and then on days 8 and 16 post-challenge and the antibody titers were determined as described in the legend for **Figure 4.5**. (b) Virus neutralizing antibodies in serum, expressed as a 50% endpoint using 100 PFU of BoHV-1, were measured on the day of challenge and on days 8 and 16 post-challenge. (c) The numbers of IFN-γ-secreting cells were measured by ELISPOT assay (as described in the legend for **Figure 4.5** on day 8 post- challenge. Bars represent the median values of each group with interquartile range.. Significant differences between groups are indicated on each graph where *p<0.05, **p<0.01 and ***p<0.001.

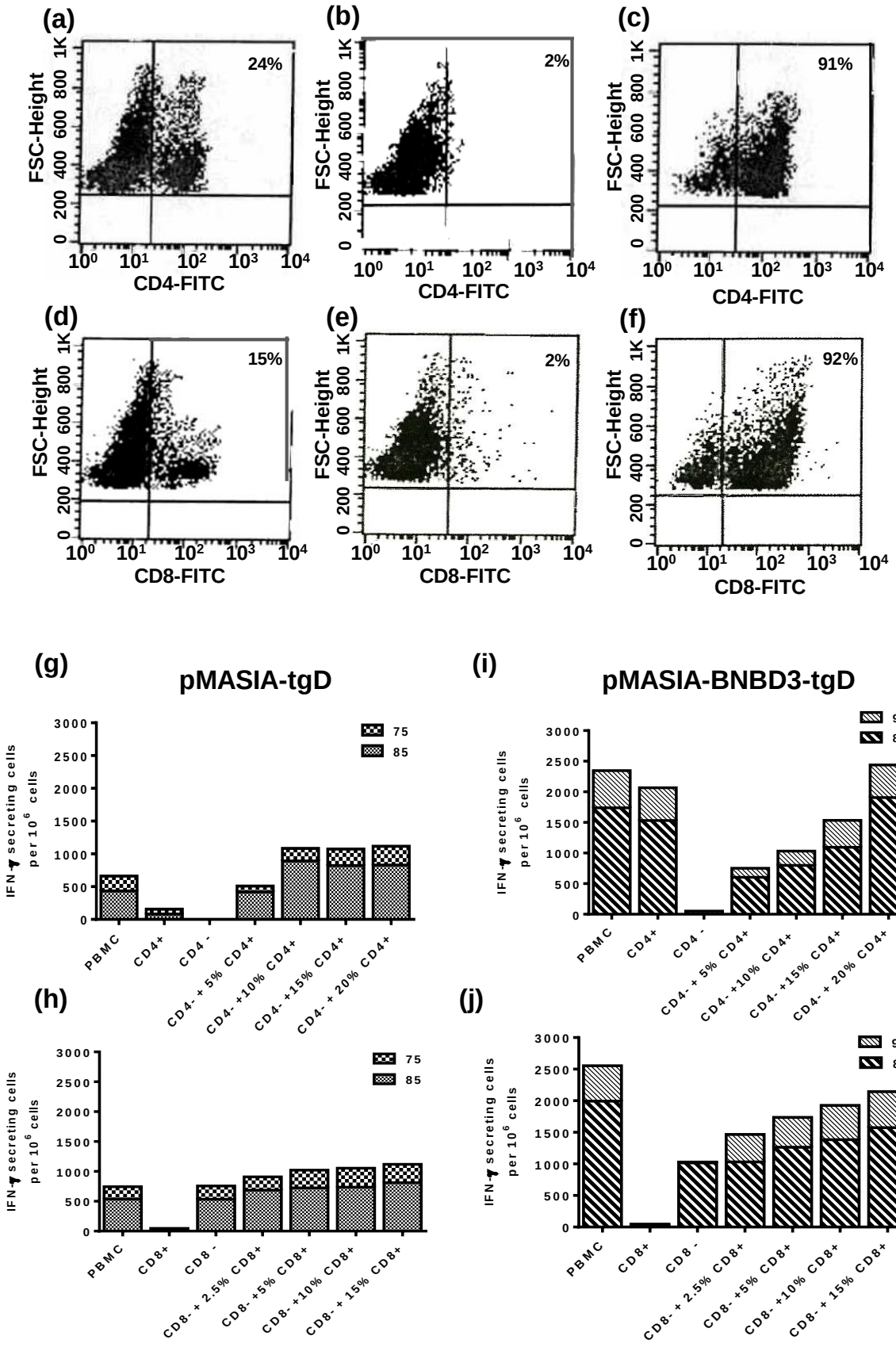
(a)
24%
(b)
2%
(c)
91%
(d)
15%
(e)
2%
(f)
92%
FSC-Height
CD4-FITC
CD8-FITC
(g)
pMASIA-tgD
(i)
pMASIA-BNBD3-tgD
(h)
(j)
IFN-γ secreting cells per 10^6 cells
75
85
96
86
PBMC
CD4+
CD4 -
CD4- + 5% CD4+
CD4- +10% CD4+
CD4- +15% CD4+
CD4- + 20% CD4+
CD8+
CD8 -
CD8- + 2.5% CD8+
CD8- +5% CD8+
CD8- +10% CD8+
CD8- + 15% CD8+

Figure 4.7 Effect of vaccination on the phenotypes of IFN-γ-secreting T-cell subpopulations in PBMCs of calves vaccinated with plasmids encoding tgD and/or-BNBD3, and challenged with BoHV-1. PBMCs were isolated from the peripheral blood of two animals from each of the pMASIA-tgD, pMASIA-BNBD3-tgD and negative control groups prior to challenge and on days 16 and 25 after challenge (2 animals per group). CD4-depleted ($CD4^-$), CD8-depleted ($CD8^-$), $CD4^+$ and $CD8^+$ subsets were isolated from PBMCs by MACS and the homogeneity of the resulting $CD4^{+/-}$ and $CD8^{+/-}$ cell populations was determined by FACS. (a-f) Dot plots are for one animal (96) from the pMASIA-BNBD3-tgD group on day 16 post-challenge and are representative of all depletions. (a) PBMCs recognized by CD4 mAb. (b) $CD4^-$ population recognized by CD4 mAb. (c) $CD4^+$ population recognized by CD4 mAb. (d) PBMCs recognized by CD8 mAb. (e) $CD8^-$ population recognized by CD8 mAb. (f) $CD8^+$ population recognized by CD8 mAb. (g,i) Frequencies of IFN-γ-secreting cells in the PBMCs, $CD4^+$, $CD4^-$, and $CD4^- + CD4^+$ (5, 10, 15, 20% of 10^6/well) cells of 2 animals from the (g) pMASIA-tgD vaccinated group and from the (i) pMASIA-BNBD3-tgD vaccinated group. (h,j) Frequencies of IFN-γ-secreting cells in the PBMCs, $CD8^+$, $CD8^-$, and $CD8^- + CD8^+$ (2.5 ,5, 10, 15% of 10^6/well) cells of 2 animals from the (h) pMASIA-tgD vaccinated group and from the (j) pMASIA-BNBD3-tgD vaccinated group. The number of IFN-γ –secreting cells per 10^6 cells was calculated as the difference between the number of spots in the tgD-stimulated wells and the number of spots in the medium-control wells. FSC=forward scatter.

To further characterize the effect of BNBD3 on the cellular immune response, an ELISPOT depletion assay was employed whereby the T cell subsets responsible for secreting IFN-γ were identified by isolating the $CD4^+$, $CD4^-$, $CD8^+$ and $CD8^-$ T cell populations from the PBMCs of two calves from each of the groups vaccinated with pMASIA-tgD, pMASIA-BNBD3-tgD or pMASIA. Typical proportions of the T cell subsets in PBMCs were 24% $CD4^+$ cells and 15% $CD8^+$ cells (Figure 4.7a,d). The purity of the isolated populations was 98% for $CD4^-$ and $CD8^-$ (Figure 4.7b,e) and >90% for $CD4^+$ and $CD8^+$ (Figure 4.7c,f). Notably, we observed five-fold higher numbers of IFN-γ secreting cells in the PBMCs from the calves vaccinated with pMASIA-BNBD3-tgD (Figure 4.7i,j) when compared to calves vaccinated with pMASIA-tgD (Figure 4.7g,h), suggesting that BNBD3 had a positive influence on the magnitude of the IFN-γ response. The $CD4^+$ T cell-depleted PBMCs from calves in the pMASIA-tgD (Figure 4.7g) or

pMASIA-BNBD3-tgD (Figure 4.7i) groups did not secrete IFN-γ in response to restimulation with tgD. For both groups, when 5, 10, 15 or 20% $CD4^+$ T cells were added to the $CD4^+$ -depleted PBMCs, IFN-γ secretion increased dose-dependently to an amount equal to that of the undepleted PBMCs (Figure 4.7g,i). Depletion of $CD8^+$ T cells did not affect the number of IFN-γ secreting cells in the PBMCs from animals in the pMASIA-tgD group, and there was also no change when 2.5, 5, 10, or 15% $CD8^+$ T cells were added to the $CD8^+$ -depleted PBMCs (Figure 4.7h). In contrast, when PBMCs of the pMASIA-BNBD3-tgD group were depleted of $CD8^+$ T cells, a two-fold reduction in the number of IFN-γ secreting cells was observed and there was a dose-dependent increase when 2.5, 5, 10, or 15% $CD8^+$ T cells were added to the $CD8^+$ -depleted PBMCs (Figure 4.7j). These depletion studies showed that $CD4^+$ T cells in the PBMCs of calves from both vaccinated groups produced IFN-γ, but that only calves in the pMASIA-BNBD3-tgD vaccinate group produced IFN-γ-secreting $CD8^+$ T cells. Thus, the addition of BNBD3 to tgD as a fusion construct appeared to increase the magnitude of the IFN-γ response, and to induce tgD-specific $CD8^+$ T cells.

To determine whether these $CD8^+$ T cells might be CTLs, the PBMCs from the same calves were restimulated with tgD and then evaluated by flow cytometry. Using a multicolor flow cytometry assay cells were identified as CTLs that, upon restimulation with the recall antigen tgD, simultaneously secreted IFN-γ and expressed CD8 and the alpha-subunit of the high-affinity interleukin-2 receptor (IL-2Rα) on the cell surface; also known as the cell activation marker, CD25 [413-416]. Within the $CD8^+$ T cell population, the percentage of cells positive for IFN-γ secretion was almost two-fold higher at 36.5% and 36.4%, after challenge in the calves

Table 4.2 Flow cytometric analysis of bovine $CD8^+$ $IFN\gamma^+$ and $CD8^+$ $IFN\gamma^+$ $CD25^+$ CTLs

		% CTLs	
Animal no.	Treatment	$CD8^+$ $IFN\gamma^+$	$CD8^+$ $IFN\gamma^+$ $CD25^+$
75	pMasia-tgD	19.7	12.5
85	pMasia-tgD	16.3	6.9
86	pMasia-BNBD3-tgD	36.5	22.1
96	pMasia-BNBD3-tgD	36.4	22.7

vaccinated with pMASIA-BNBD3-tgD than the 19.7% and 16.3% positive cells observed for calves vaccinated with pMASIA-tgD (Table 4.2). This increased IFN-γ secretion by $CD8^+$ T cells correlated well with, and may account for some of the higher numbers of IFN-γ producing cells observed in, the ELISPOT results (Figure 4.6c, Figure 4.7i,j) from calves in the pMASIA-BNBD3-tgD group. To identify CTLs within this population co-expression of the CD25 activation marker by these $CD8^+$ IFN-γ^+ T cells was examined. The percentage of $CD8^+$ IFN-γ^+ $CD25^+$ cells from calves vaccinated with pMASIA-BNBD3-tgD was 22.1% and 22.7%, representing a greater than two-fold increase as compared to the 12.5% and 6.9% of $CD8^+$/ IFN-γ^+ $CD25^+$ cells observed for calves vaccinated with pMASIA-tgD (Table 4.2). Thus, in good agreement with the results in mice, in cattle the addition of BNBD3 as a fusion construct with tgD increased the population of what are considered CTLs, namely activated, tgD-specific $CD8^+$ T cells. No effect of vaccination with the BNBD3-encoding vaccine on the numbers of γδ+/IFNγ+/CD25+ T cells was observed.

These results demonstrate that in vaccinated, BoHV-1 challenged cattle the addition of BNBD3 as a fusion construct increased the number of IFN-γ secreting cells, specifically the number of IFNγ-secreting tgD-specific $CD8^+$ T cells, and notably the $CD8^+$ IFN-γ^+ $CD25^+$ subset.

4.4.5 Clinical observations of protection after BoHV-1 challenge

Calves in all three groups shed virus from day 2 after challenge, with significantly less virus shed on day 8 by calves in the pMASIA-BNBD3-tgD ($p<0.01$) and pMASIA-tgD ($p<0.05$) groups when compared to the pMASIA group (Figure 4.8a). By day 10, both the pMASIA-BNBD3-tgD and pMASIA-tgD groups showed equal and significantly less viral shedding ($p<0.05$) when compared to the pMASIA group (Figure 4.8a). Weight loss was observed in calves from all three groups by day 2 to day 4 after challenge (Figure 4.8b). On day 6, weight loss was reversed for both the pMASIA-BNBD3-tgD and pMASIA-tgD groups, whereas calves in the pMASIA group continued to lose weight. Additionally, on day 6 only calves in the pMASIA-BNBD3-tgD ($p<0.05$) group had significantly less weight loss when compared to the pMASIA group, while by day 10 calves in both pMASIA-BNBD3-tgD and pMASIA-tgD groups had equal and significantly less weight loss ($p<0.05$) when compared to calves in the pMASIA group (Figure 4.8b). Rectal temperatures peaked for all three groups on day 2 after challenge and then began falling (Figure 4.8c). Between days 8 and 10, temperatures rose in the pMASIA group, while they fell in the pMASIA-BNBD3-tgD and pMASIA-tgD groups.

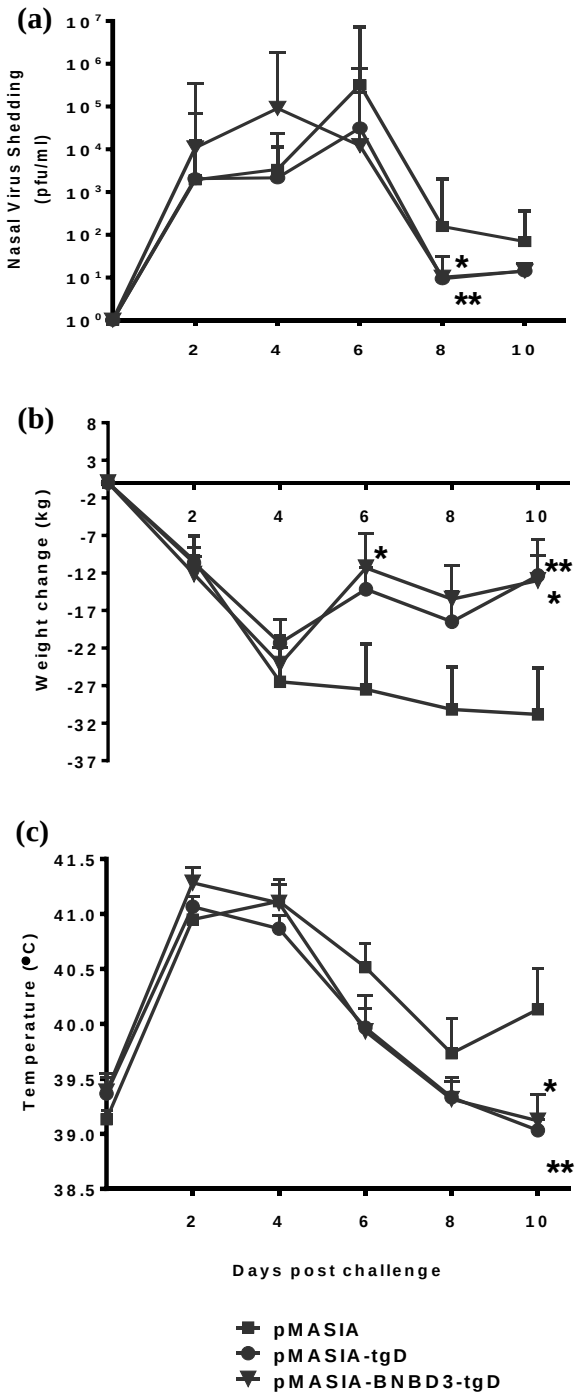

Figure 4.8 Clinical signs and virus shedding after BoHV-1 challenge. Eight to nine month-old BoHV-1 seronegative Angus and Hereford crossbred calves were immunized three times ID by needle-free injection with 0.75 mg plasmid followed by BoHV-1 challenge one month after the last immunization (6 animals per group). (a) Mean (geometric) virus shedding in nasal secretions of calves challenged with BoHV-1. (b) Mean weight change. (c) Mean rectal temperatures. *$p<0.05$; **$p<0.01$.

By day 10 after challenge, rectal temperatures were significantly lower in calves from the pMASIA-BNBD3-tgD ($p<0.05$) and pMASIA-tgD ($p<0.01$) groups than in those from the pMASIA group (Figure 4.8c). These results demonstrate that in cattle the addition of BNBD3 as a fusion construct was able to protect cattle from infection with BoHV-1 equally as well, but not better than, the control DNA vaccine, pMASIA-tgD.

4.5 Discussion

In this study the effect of DNA vaccines encoding an iDC chemotactic peptide BNBD3, separately or as a fusion construct with BoHV-1 tgD, on the immune responses was determined in mice and cattle. In mice, addition of pMASIA-BNBD3 to pMASIA-tgD had no effect on tgD-specific IgG and increased the number of IFN-γ secreting cells, while in cattle this two-plasmid treatment did not improve either humoral or cellular immune responses. When BNBD3 was delivered with tgD as a fusion construct in pMASIA-BNBD3-tgD, again there was no significant effect on tgD-specific antibody production; however, the CMI responses were enhanced in both mice and cattle. The addition of BNBD3 as a fusion with tgD induced a greater number of IFN-γ secreting $CD8^+$ T cells. In mice, this construct induced increased numbers of $CD8^+$ CTLs, and interestingly also a population of cells identified as non-T ($CD3^-$)/$IFN\gamma^+$/$CD8^+$ that may have been $CD8\alpha^+$ splenic DCs. In cattle, $CD8^+$/IFN-γ^+ cells were increased and $CD8^+$/IFN-γ^+/$CD25^+$ CTLs were induced only in animals immunized with pMASIA-BNBD3-tgD. However, despite the fact that immunization with pMASIA-BNBD3-tgD enhanced the CMI response, protection from challenge was similar to that provided by immunization with pMASIA-tgD.

Like our results in cattle, in earlier studies in mice mixtures of free, unlinked murine chemokines and antigen [391], or a mixture of plasmids expressing unlinked antigen and murine β-defensin [217], did not induce an immune response [217, 218, 391]. In our study, segregation of BNBD3 and tgD either physically or temporally may have occurred in cattle and not in mice; this phenomenon of separation has been suggested previously to account for little or no effect when plasmids are mixed [420]. Additionally, it has been noted that responses to mixtures of plasmids can result in interference, leading to dominant Th2 responses, less appropriate responses [420], or suppression of responses [421]. The outbred nature of cattle as a species might have also contributed to the lack of effect of BNBD3 in cattle when delivered as a separate plasmid, as immune responses to mixtures of plasmids have been found to be lower in outbred mice than in inbred mice [422].

Inclusion of BNBD3 in the DNA vaccine as a fusion construct pMASIA-BNBD3-tgD did not affect humoral responses to tgD, but increased CMI responses and appeared to strengthen the Th1 bias in mice. Similarly, in cattle this vaccine was unable to improve serum antibody levels, but it increased proliferation of PBMCs and the number of IFN-γ secreting cells. Our results are comparable with the findings of an earlier study in mice, where the ability of an analogous fusion construct comprised of mBD2 (and mBD3) in combination with idiotypic antigen (Id) expressed by malignant B cells, to induce protective and therapeutic immunity to lymphoma was tested [217]. sFv, a single chain Ig made up of the linked Vh and Vl domains of the Fv fragment of the Ig receptor of the malignant B cell (described in [391]) failed to elicit an Id-specific antibody response when the sFv-encoding DNA vaccine was delivered alone or with mBD3 on a separate plasmid, whereas responses were observed after vaccination with fusion constructs of either

mBD2, mBD3, mMIP3α or mSLC [217]. Protective immunity against an aggressive lymphoma (38C13) was obtained after DNA vaccination by both mBD-sFv fusion plasmids even though the humoral response was considerably lower with the vaccine encoding mBD2. The authors concluded that fusion of tumor antigen with a chemokine or defensin that targets iDCs was important for both tumor prevention and eradication. While humoral immunity contributed to protection from tumors, cellular antitumor immunity was necessary for both protection and therapeutic antitumor immunity [217]. In contrast to our results, this study suggests that the increased CMI responses induced by the defensin-antigen fusion was critical to antitumor efficacy.

In mice the fusion construct pMASIA-BNBD3-tgD modulated the cellular immune response by inducing $CD8^+$/IFN-γ^+/ $CD3^+$ CTLs and a population of cells that were $CD8^+$/IFNγ^+ but that were not T cells. It is possible that these $CD8^+$/IFN-γ^+/$CD3^-$ cells may have been DCs, since splenic $CD8\alpha^+$ DCs have been described. This type of DC lacks expression of CD3 [419] and is a potent secretor of IFN-γ where IFN-γ is produced in an autocrine manner in response to IL-12 secreted by the cells exposed to a bacterial stimulus [423]. These DCs are expanded in response to signals from the innate immune system as a result of bacterial or viral infection [423-425]. They cross-prime [426], prime [425] or prime and boost $CD8^+$ T cell responses and activate memory $CD8^+$ T cells [427], trigger the development of Th1-type cells/response [428, 429], and cause apoptotic death of activated $CD4^+$ T cells [430]. As defensins are innate immune system molecules, BNBD3 might have influenced induction of $CD8\alpha^+$ DCs in the same manner as they are increased by bacterial/viral infection, though this was not proven by this study. Since $CD8\alpha^+$ DCs preferentially prime CTLs this would explain the increased CTL response we observed.

Although the existence of these cells as a result of DNA immunization with β-defensin-antigen fusion constructs has not been reported yet, the presence of such cells would clarify many of the hitherto unexplained findings of ourselves and others.

In vaccinated and BoHV-1 challenged cattle the addition of BNBD3 as a fusion construct modified the immune response; VN antibody levels were maintained, and the number of tgD specific IFN-γ-secreting cells, particularly $CD8^+$/IFN-γ^+ cells and $CD8^+$/IFN-γ^+/$CD25^+$ CTL cells, were increased. Thus, BNBD3 promoted a predominantly Th1 response that included induction of $CD8^+$ CTLs. While it is generally accepted that Th1 immune responses drive cellular immunity and Th2 immune responses preferentially drive humoral immunity [431, 432], and indeed the Th1-polarized/biased cellular response that we observed fits neatly into this model, in this context, it was puzzling that the VN antibody was maintained. Our data may be explained however, by the findings of others. Reports have suggested that while Th1 type cytokines exert an overall negative effect on systemic humoral responses [431, 433], they can have a positive effect on the magnitude of neutralizing antibody response [434] and that neutralizing antibody responses can occur concurrent with induction of Th1 polarized responses [435-437]. In particular, our results (VN antibody concurrent with induction of CTL) with this defensin-antigen fusion construct in cattle bears striking similarity to the results obtained when in the mouse model, an experimental DNA vaccine expressing murine β-defensin 2 (mBD2) as a fusion with the gp120 antigen of HIV-1 induced systemic and mucosal CTL and neutralizing antibody to the HIV-1 envelope protein in ID immunized mice [218]. Although the exact mechanism was not determined, the authors theorized that the immunomodulatory effect of the vaccine could have been due to the previously discovered chemotactic nature of mBD2 for iDCs

[391]. Additionally, the authors suggested that β-defensin might have targeted receptors on APCs, induced expression of costimulatory molecules and/or induced production of proinflammatory cytokines particularly by iDCs [218].

More recently, a β-defensin adjuvanting strategy was evaluated in chickens whereby birds were immunized intramuscularly with a DNA vaccine encoding a fusion construct of the mature form of avian β-defensin 1 (AvBD1) with the VP2 protein of infectious bursal disease virus (IBDV) [388]. Unlike our results in cattle, in this study the plasmid encoding the fusion construct induced significantly greater antibody responses than the plasmid encoding the antigen (VP2) alone. The greater antibody response might have been due to the route of administration, as higher humoral responses have been observed when DNA vaccines have been delivered IM [438], or to differences in species or the two β-defensins. Comparable to the augmented cellular responses including increased numbers of $CD8^+$ cells that we observed in cattle, in this avian model increased percentages of CD3, CD4 and CD8 T cells were observed in birds immunized with the fusion construct. After challenge with IBDV the ten chickens immunized with the AvBD1 fusion construct were protected, while in the group given the DNA vaccine encoding VP2 alone, eight out of ten were protected. Despite the modest improvements in humoral and cellular immunity and protection from IBDV, the authors concluded that AvBD1 in a fusion construct enhanced VP2-DNA vaccine immunity and protection from IBDV. The authors further suggested that the effect of AvBD1on improved CMI responses may have been responsible for the protection induced by the fusion construct; particularly since CMI and specifically T cell responses had been shown to be important in protection from IBDV infection.

In cattle, protective vaccination against BoHV-1 has been described for commercially available MLV or KV BoHV-1 vaccines. As such it has been defined as an observed reduction in clinical signs such as decreased virus shedding, lowered temperature and decreased nasal secretions [439]. Following challenge with BoHV-1, we observed a reduction in the clinical signs of infection in calves vaccinated with the fusion construct pMASIA-BNBD3-tgD. Contrary to what was observed in the avian model, the addition of β-defensin gave protection equivalent to, but not better than, what was observed in the group given the DNA vaccine encoding the antigen alone. This was surprising in light of the improvements seen in the avian study, and because protective vaccination against BoHV-1 has also been associated with increased CMI responses, particularly those in the form of increased IFN-γ production [439]. Given that humoral immunity was not enhanced in calves vaccinated with pMASIA-BNBD3-tgD when compared to those vaccinated with pMASIA-tgD, and that inefficient humoral immune responses have been implicated in a lack of protection from BoHV-1 challenge (reviewed in [440]), this does suggest that the humoral immune responses were not high enough and that the improved cellular immunity induced by BNBD3 was not sufficient to result in enhanced protection from BoHV-1. Here we tested our hypothesis that inclusion of an iDC chemotactic β-defensin either encoded on a separate plasmid or as a fusion construct with antigen in a DNA vaccine, would improve efficacy of a BoHV-1 DNA vaccine for cattle. In summary, delivery of BNBD3 by separate plasmid did not enhance immune responses in cattle, while the addition of BNBD3 as a fusion construct modulated the immune response to the DNA vaccine resulting in increased cell-mediated immunity. Protection against BoHV-1 was afforded to an equal extent by DNA vaccines encoding tgD alone or as a fusion with BNBD3. Taken together, from our results and those of others regarding the effect of β-defensins on DNA vaccines, some patterns emerge that

are worth noting as they suggest directions where further study could be productive. With respect to humoral responses, systemic antigen-specific IgG responses appear to vary with the antigen, the route of delivery, and the species, while CMI responses appear to be improved by beta-defensin regardless of the nature of these factors.

5. EFFECT OF COMPLEXES OF BOVINE HERPESVIRUS-1 (BOHV-1) GLYCOPROTEIN D DNA VACCINE WITH CATIONIC BOVINE NEUTROPHIL BETA-DEFENSIN 3 ON IMMUNE RESPONSES OF MICE

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Keywords: Herpesvirus, host defense peptide, defensin, adjuvant, DNA vaccine, immune responses

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Fifth Chapter Transition/Overview

In the previous chapter/manuscript (chapter 4) I tested my hypothesis that inclusion of the iDC chemotactic β-defensin BNBD3, either encoded on a separate plasmid or as a fusion construct with tgD, would improve efficacy of a BoHV-1 DNA vaccine for cattle. Although the pMASIA-BNBD3-tgD fusion construct vaccine increased CMI and afforded protection against BoHV-1, the vaccine was unable to improve clinical responses of BoHV-1 challenged cattle. I hypothesized that the lack of improvement in clinical responses may have been because the vaccine failed to improve the humoral response. Thus in this chapter I suggest that an ideal DNA vaccine strategy for BoHV-1 would increase humoral responses and maintain robust CMI. Accordingly, for the third objective I evaluated in mice a strategy that utilizes BNBD3 in its peptide form, complexed with the DNA vaccine pMASIA-tgD, for its potential to improve humoral immune responses while maintaining robust CMI. Vaccination with a complex comprised of pMASIA-tgD complexed to 0.1875 nmol aBNBD3 (125:1 nanomolar ratio) resulted in improved Th1 type humoral response and maintained the strong CMI response relative to pMASIA-tgD. This improvement may have occurred through chemotaxis of iDCs. Additionally, this improvement may have occurred through the effect of BNBD3 in the complexed vaccine on murine BMDCs as *in vitro* studies of the immunomodulatory effects of BNBD3 showed that BNBD3 activated and functionally matured these cells. Since both robust antibody and CMI responses of a Th1 type are desired for protection from BoHV-1 infection, and this strategy does result in both, the results of this chapter support my overall hypothesis that inclusion of an iDC chemotactic bovine β-defensin in a DNA vaccine can lead to strong humoral and CMI responses.

5.1 Abstract

Protective efficacy against BoHV-1 afforded through increased cell-mediated immune responses was recently demonstrated by a DNA vaccine encoding the DC-chemotactic bovine beta-defensin, BNBD3 as a fusion with the viral antigen tgD. However the vaccine was unable to improve clinical responses of BoHV-1 challenged cattle over what was observed in animals vaccinated with the DNA vaccine encoding the tgD antigen alone and this may have been because the vaccine failed to improve the humoral response. We hypothesized that an alternative vaccine design strategy that utilized the DNA vaccine pMASIA-tgD as a complex with the cationic peptide BNBD3 might be able to improve the humoral response while maintaining a robust Th1-type cell-mediated response. C57Bl/6 mice were vaccinated twice intradermally with pMASIA-tgD complexed with 0, 0.01875, 0.1875, or 1.875 nmol of a stable synthesized analog of BNBD3 (aBNBD3). The best results were seen in mice immunized with the vaccine comprised of pMASIA-tgD complexed to 0.1875 nmol aBNBD3. In this group the humoral response was improved as evidenced in increased tgD-specific IgG2a, while the strong CMI response, measured based on specific IFN-γ-secreting cells, was maintained relative to pMASIA-tgD. Modulation of the immune response may have been due in part to the effect of BNBD3 on dendritic cells (DC). *In vitro* studies showed that murine bone marrow-derived DC (BMDC) pre-treated with aBNBD3 were activated as evidenced by down-regulated CD11c and were functionally matured as shown by increased allostimulatory ability. Native, synthesized or analog BNBD3 were equally capable of inducing functional maturation of BMDCs.

5.2 Introduction

Bovine herpesvirus-1 (BoHV-1) is an economically important veterinary pathogen. Like other alphaherpesviruses such as herpes simplexvirus-1,2 (HSV-1,2/HHV-1,2) in humans [441], pseudorabies virus (PrV/SuHV-1) in pigs and equine herpesvirus-1,4 (EHV-1,4) in horses, the initial infection with BoHV-1 is typically followed by the establishment of viral latency [18, 57, 59]. Latency represents the ultimate example of immune-modulation of the host by a virus, as once latency is achieved, the virus can be reactivated many times throughout an animal's productive lifetime causing recurrent infection, viral shedding, and spread of the virus to new hosts (naïve animals) [87, 442]. Along with hygienic measures, it's quite sensible to seek methods that prevent infection in the first place, and since vaccination is a primary method of prevention, it follows that development of an effective non-infectious preventative vaccine would be desirable.

DNA vaccines are non-infectious. Additionally, they are simple in design and economical to produce thus making them attractive as veterinary vaccines [242]. Immunization with a DNA vaccine results in endogenous host-cell expression of the antigen, with a subsequent antigen-specific immune response [241, 443]. In mice this results in a predominantly Th1-type response with induction of IgG2a isotype antibody and CTL [444]. Additionally, antigen expressed by the host cells can be picked up by infiltrating APCs, such as DCs [269] or circulated as free antigen to stimulate humoral responses [445]. DNA from immunization can also be found in directly transfected APCs [262], as free DNA in draining lymph which can then transfect DCs in regional LNs [262, 446], and in transfected MΦs in the peripheral blood [447]. A major hurdle to

development of a DNA vaccine for herpesviruses in general and for BoHV-1 in particular, is that both cell-mediated and humoral responses are required [67] and need to be increased over that which can be obtained with naked DNA [399]. To achieve improvements in both arms of the immune response has proven to be a challenge and has led to many studies with attempts at various immune enhancing strategies including genetic adjuvanting [217, 218, 448], delivery by liposomes [449, 450], and adding or complexing adjuvants to the plasmid DNA [444, 451].

Recently, we evaluated the potential of a genetic adjuvanting strategy for the BoHV-1 DNA vaccine, pMASIA-tgD. In cattle a DNA vaccine encoding the DC-chemotactic bovine β-defensin BNBD3 as a fusion with the viral antigen tgD showed protective efficacy against BoHV-1 through increased Th1-type cell-mediated responses [452]. β-defensins are cationic, membrane active, antimicrobial proteins of the innate immune system that participate in defence against microbiological pathogens [199]. They are small peptides, 38 to 42 amino acids in length, characterized by an N-terminal α-helix and six conserved cysteine residues that form three disulfide bonds defined as Cys1-Cys5, Cys2-Cys 4, Cys3-Cys6 [175, 199, 287]. In cattle, sixteen β-defensins have been discovered. Thirteen are produced by neutrophils and are known as bovine neutrophil β-defensins 1-13 (BNBD1-13) of which BNBD3 is the most abundant [219]. The vaccine was unable to improve clinical responses of BoHV-1 challenged cattle over what was observed in animals vaccinated with the DNA vaccine encoding the tgD antigen alone however, and this may have been because the vaccine failed to improve the humoral response.

We hypothesized that an alternative vaccine design strategy that utilized the DNA vaccine pMASIA-tgD as a complex with the cationic peptide BNBD3 might be able to improve the

humoral response while maintaining a robust Th1-type cell-mediated response. Our hypothesis was based on the finding by Reidle et al. (2004) that when a small cationic peptide fused to a short antigenic epitope was complexed with a DNA vaccine encoding for a full length antigen, the humoral immune response to the DNA-encoded antigen could be improved without loss of CMI responses; but only when the two components were complexed at the low peptide to DNA ratio of 125:1 [453, 454]. This phenomenon was not related to improved uptake of the DNA or subsequent expression of the antigen encoded by the DNA and the authors were unable to explain the mechanism, or whether and how it might be related to the cationic nature of the peptide.

Enhanced serum antibody responses without loss of CTL have also been observed when the DNA complexing adjuvant has been in the form of a cationic microparticle [455] cationic emulsion [444] or contained a cationic lipid such as DOTAP [449]. Additionally, it was found that although macrophages could act as APCs after exposure to naked DNA, DCs could only be transfected with plasmid DNA and subsequently act as APCs when transfected in the presence of cationic lipid [456]. Thus there does appear to be a benefit when the DNA complexing adjuvant has been of a cationic nature.

Modifying the fundamental Th1 cellular response to plasmid DNA vaccination with the BoHV-1 antigen gD [399] to that of an improved humoral response could, however, be difficult. In a murine model of HSV-2/HHV-2, the gD antigen when delivered as a protein was a strong inducer of Th2 responses with high antibody titers, whereas the same antigen when delivered by DNA vaccine induced a response with a Th1 bias [431, 457]; a bias that was strengthened when

genetically adjuvanted with plasmid encoding the Th1 cytokine IL-12 [431, 458] and that could not be redirected by genetic adjuvanting with plasmid encoding Th2 cytokines IL-4 or IL-10 [458].

Since the use of a bovine β-defensin as the cationic component in a peptide/DNA complexed BoHV-1 vaccine as a way to enhance humoral responses has not been studied, in this work we evaluated the potential of this strategy. The DNA vaccine pMASIA-tgD was complexed with increasing amounts of the cationic peptide aBNBD3 and the complexed vaccine was studied for its capacity to stimulate immune responses in mice *in vivo*, and by BNBD3 for effects on murine BMDCs *in vitro*. The best results were seen in mice immunized with the vaccine comprised of pMASIA-tgD complexed with aBNBD3 at the medium nanomolar peptide to DNA ratio of 125:1. Immunization with this complexed vaccine maintained robust Th1-type CMI, induced tgD-specific IgG, IgG1, and significantly improved IgG2a antibody responses. *In vitro* studies revealed that aBNBD3 activates and functionally matures murine BMDC.

5.3 Materials and Methods

5.3.1 Cationic peptides/BNBDs and plasmids

BNBD3 peptides were chemically synthesized, and then folded to form the defined β-defensin disulfide connectivities (Cys 1-5, Cys 2-4, Cys 3-6) as described [389]. Native BNBD3, isolated from the bovine neutrophil [219] was kindly provided by Dr. Micheal Selsted (University of California, Irvine, CA, United States). The amino acid sequences of the synthesized peptides sBNBD3 (synthesized based on the published sequence with Glutamine [Q] in amino acid positions 1 and 27 (UniprotKB/Swiss-Prot accession number P46161)) and aBNBD3 (an analog

synthesized such that Glycine [G] replaced Glutamine [Q] in amino acid positions 1 and 27) are shown in relation to native BNBD3 and to human and murine β-defensin 2 (Figure 5.1).

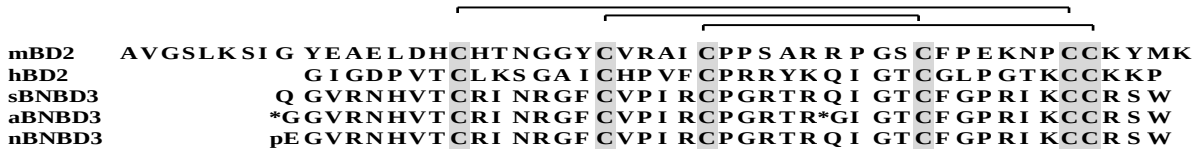

Figure 5.1 Amino acid sequence alignment of native and synthesized forms of BNBD3. sBNBD3 (published sequence UniprotKB/Swiss-Prot accession number P46161- Glutamine [Q] in positions 1 and 27), aBNBD3 (analog, Glycine [G] replaces Glutamine [Q] in positions 1 and 27), nBNBD3 (the sequence of Native BNBD3; pyroglutamic acid [pE] the modified residue of Glutamine [Q] at N-terminus), aligned with murine(m)BD2, and human(h)BD2. Aligned conserved cystiene residues are shown in shaded vertical bars and the β-defensin disulfide connectivities (Cys 1-5, Cys 2-4, Cys 3-6) are shown at the top.

The plasmids pMASIA and pMASIA-tgD, which encodes truncated glycoprotein D (tgD) of bovine herpesvirus-1 (BoHV-1), have been detailed previously [452]. These plasmids were amplified in *Escherichia coli* JM109 cells, purified with Endofree Plasmid Giga kits (Qiagen; Montreal, QC, Canada), suspended as stock solutions in water (3.12-3.5 mg/ml), and stored at -20°C. Their application/use in cattle and mice as DNA vaccines has been described [452].

5.3.2 Chemotaxis assay

Biological activity of the synthesized BNBD3s was confirmed using a chemotaxis assay described previously [389]. Briefly, chemotaxis of bovine iDC to synthesized BNBD3 was compared to native BNBD3 using a 96-well disposable chemotaxis system (ChemoTx system; Neuroprobe, Gaithersburg, MD, USA). Peptides diluted to 1000, 100, 10, 1, 0.1, 0.01 or 0

(buffer) ng/ml were placed in triplicate wells of the bottom chamber of the plate. Bovine Mo were isolated from peripheral blood mononuclear cells (PBMCs) of four donor animals and were cultured with supernatants from bovine IL-4 and bovine GM-CSF transfected CHO cells (kindly provided by Merial Limited, Lyon, France) for 3 days to generate iDCs as verified by FACs analysis. The monocyte-derived iDCs were labelled with Calcein AM (Molecular Probes), placed on the top of the membrane above each BNBD3-or buffer-filled well and the plates were incubated for 90 min at 37 °C. Migrated cells were identified using a 96-well multilabel plate reader (Victor 3V Multilabel Counter, PerkinElmer Life And Analytical Sciences, Inc., Woodbridge, ON, Canada). Chemotactic index (CI) was calculated for each well by dividing the total fluorescence of each test well by the mean fluorescence of the buffer/control wells. A CI $\geq$ 2 is considered statistically significant ($p < 0.05$) [315].

5.3.3 Preparation of BNBD3-DNA complexed vaccines

Cationic peptide/DNA complexed vaccines were prepared according to the method described by Riedle et al. (2004) [454]. Accordingly, pMASIA-tgD (5 µg / 0.0015 nmol) was mixed with 0.01875 nmol (Low), 0.1875 nmol (Medium), or 1.875 nmol (High) of the cationic peptide aBNBD3 in a Ca/Mg free PBS buffer (pH 7.4; Gibco) in a final volume of 20 µl for 60 min at RT. The nanomolar ratio of peptide to DNA was thus 12.5:1 for the low dose, 125:1 for the medium dose and 1250:1 for the high dose. Complex formation of DNA and peptide was visualized by agarose gel electrophoresis in an electrophoretic mobility shift assay (EMSA). Samples were loaded in 10% glycerol, and were then applied to a 1% agarose gel containing 0.5 µg/ml ethidium bromide and run for 90 min at 50V in TAE running buffer (40mM Tris-acetate, 1mM EDTA; pH 8.3).

5.3.4 Immunization of mice with BNBD3-DNA complexed vaccines

To test the ability of BNBD3, delivered as a complex with the tgD-encoding DNA vaccine pMASIA-tgD, to enhance tgD-specific humoral responses without reduction of specific cellular responses, six- to eight-week old C56BL/6 mice (n=8) were immunized twice at a 4 week interval. Immunizations were delivered as 2 x 10 µl intradermal injections into the base of the tail. Each 20 µl dose was formulated to contain 5µg pMASIA (placebo), 5µg pMASIA-tgD, 5 µg pMASIA-tgD (0.0015 nmol) plus 0.01875 nmol BNBD3 (Low), 5 µg pMASIA-tgD (0.0015 nmol) plus 0.1875 nmol BNBD3 (Medium), or 5 µg pMASIA-tgD (0.0015 nmol) plus 1.875 nmol BNBD3 (High). One month after the final vaccination, mice were euthanized. Serum was taken to be assayed for tgD-specific antibody levels and spleens were collected for analysis of tgD-specific CMI responses.

5.3.5 Enzyme-linked-immunosorbent assay (ELISA)

To measure tgD-specific IgG, IgG1, and IgG2a responses, 96-well polystyrene microtiter plates (IMMULON® 2; Thermo Electron Corrp., Milford, MA) were coated overnight with 0.05 µg of tgD per well in sodium carbonate coating buffer and then washed in PBS with 0.05% Tween 20 (PBST). Serum was collected from each mouse, and serial dilutions starting at 1:40 in four-fold dilution in PBS containing 0.5% gelatin (PBS-g) were dispensed to plates. Following overnight incubation at 4°C, plates were washed, and bound IgG was detected using alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (Kirkegaard & Perry Laboratories) diluted 1:5000 in PBS-g for 1 h at RT. Bound IgG1 and IgG2a were detected using biotinylated goat anti-mouse IgG1 and IgG2a antibodies (Caltag Laboratories, Burlingame, CA) diluted 1:5000 in PBS-g for 1 h at RT followed by streptavidin-AP at a dilution of 1:10,000 for 1 h at RT. All reactions were

visualized with 0.01M *p*-nitrophenyl phosphate (PNPP) (Sigma-Aldrich) in 0.104 M diethanolamine, 0.5 mM MgCl. Absorbance was read on a model 3550 Microplate Reader (Bio-Rad Laboratories Ltd) at 405 nm, with a reference wavelength of 490 nm. ELISA titers were expressed as the inverse of the serum dilution that gave an absorbance (*A*) value two standard deviations above the values for serum from control naïve animals.

5.3.6 IFN-γ and IL-5 Enzyme-linked-immunospot (ELISPOT) assay

To measure the number of IFN-γ and IL-5 secreting cells, nitrocellulose plates (96-well Multiscreen-HA; Millipore Corp., Bedford, MA, USA) were coated overnight at 4°C with 0.2 μg/well of anti-mouse IFN-γ or IL-5 (BD Biosciences, San Jose, CA, USA). Plates were washed with PBS and then blocked with 1% (w/v) bovine serum albumin (BSA; Sigma-Aldrich) for 2 h at 37°C. Splenocytes were isolated as previously described [402] and resuspended at a concentration of 1 x 10^7 cells per ml in complete medium (cRPMI) made up of RPMI 1640, supplemented with 10% fetal bovine serum (FBS), 50 μg/ml gentamycin, 1 mM L-glutamine, 10 mM Hepes, 1 mM non-essential amino acids, 1 mM sodium pyruvate (all Life Technologies Inc., Burlington, ON, Canada) and 5 x 10^{-5} M 2-mercaptoethanol (Sigma-Aldrich). Splenocytes at 10^6 cells/well were added to triplicate wells containing medium or tgD at 3 μg/ml and incubated at 37°C for 20 h. Plates were washed, and then incubated with 2 μg/ml biotinylated rat anti-mouse IFN-γ or IL-5 (BD Biosciences) in PBS with 1% BSA for 1.5 h. Bound antibodies were detected using streptavidin-AP (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted 1:1000 in PBS with 1% BSA for 1.5 h and visualized using BCIP/NBT substrate (Sigma-Aldrich). Stained spots were counted and the number of IFN-γ or IL-5 secreting cells per 1 x 10^6

cells was expressed as the difference between the number of spots in the tgD-stimulated wells and the number of spots in the control (medium) wells.

5.3.7 Murine bone marrow-derived dendritic cell (BMDC) generation and stimulation

Changes in cell surface expression of maturation markers or in proliferative ability of BMDCs were used to assess the effect of BNBD3 on *in vitro* maturation and activation of DCs. BMDCs were prepared as previously described [459], with modifications. Briefly, bone marrow cells were flushed from femurs and tibiae of naïve C57BL/6 mice, and depleted of erythrocytes by lysis with Tris-ammonium chloride buffer (17 mM Tris, 144 mM NH_4Cl, pH 7.2). Subsequently, the cells were suspended at 1 x 10^6 cells/ml in RPMI 1640 medium supplemented with 10% FBS, 50 μg/ml gentamycin, 1 mM L-glutamine, 10 mM Hepes, 1 mM non-essential amino acids, 1 mM sodium pyruvate (all Life Technologies Inc., Burlington, ON, Canada), 5 x 10^{-5} M 2-mercaptoethanol (Sigma-Aldrich) and 20 ng/ml murine GM-CSF (PeproTech Inc., Rocky Hill, NJ) (DC medium). Cell suspensions of 4 ml/well were cultured in 6-well plates with non-adherent cells (granulocytes, T and B cells) removed and fresh DC medium added on day 3. For the studies shown in Figure 5.6, on days 5, 7 and 9, 50% of the media was removed and replaced with fresh DC medium, and additionally on day 9, cells were untreated or treated for 24 h with lipopolysaccharide (LPS) at a final concentration of 100 ng/ml or with aBNBD3 at a final concentration of 10, 100 or 1000 ng/ml. On day 10, cells were harvested for FACs analysis or for use in proliferation assays. For the proliferation studies shown in Figure 5.7, BMDCs were harvested at day 5, resuspended at 1 x 10^5 cells/ml in DC medium and dispensed (100 μl of cells/well) to U-bottom 96-well tissue culture plates. Cells were untreated or treated for 18 h with LPS at a final concentration of 100 ng/ml or with nBNBD3, sBNBD3, aBNBD3 at final

concentrations of 10, 100 or 1000 ng/ml or with LPS (100 ng/ml), in combination with nBNBD3 or sBNBD3 or aBNBD3 at the above concentrations.

5.3.8 Flow cytometry analysis of stimulated BMDCs

The BMDC phenotype was examined on day 10 after 24 h incubation with the different treatments. Cells were washed and resuspended at 1 x 10^7 cells/ml in FACs buffer (PBS pH 7.2, 0.1% BSA, 0.05% $NaNH_3$) supplemented with 2% FBS, and 100 µl aliquots were cell-surface stained for 30 min at 4°C with FITC anti-mouse I-A[b] (MHCII, clone AF6-120.1, BD Biosciences), FITC anti-mouse CD11c (clone HL3, BD Biosciences), FITC anti-mouse CD40 (clone 3/23, BD Biosciences), or FITC anti-mouse CD86 (clone GL1, BD Biosciences). Staining specificity was controlled with the appropriate isotype-matched antibody controls. Cells were washed and resuspended in PBS. Samples were acquired using a FACSCalibur flow cytometer, and the data were analyzed with CELLQuest software (BD Biosciences).

5.3.9 Mixed leukocyte reaction

To assess the functional activity of BMDCs stimulated by nBNBD3, sBNBD3, or aBNBD3, allogeneic MLR assays were performed using mouse splenocytes as responder cells. Dendritic cells were cultured as described above. For Figure 5.6 the BMDCs, untreated or treated with LPS or aBNBD3, were harvested at day 10 and were resuspended in proliferation medium (PM) made up of RPMI 1640, supplemented with 1 ng/ml dexamethasone, 10% FBS, 50 µg/ml gentamycin, 1 mM L-glutamine, 10 mM Hepes, 1 mM non-essential amino acids, 1 mM sodium pyruvate (all Life Technologies Inc., Burlington, ON, Canada), 5 x 10^{-5} M 2-mercaptoethanol (Sigma-Aldrich) and dispensed to U-bottom 96-well tissue culture plates to provide 0.5, 1, 1.5 and 2 x 10^4

cells/well. For Figure 5.6, 100 µl of responder cells/splenocytes (2 x 10^6 cells/ml in PM) and for Figure 5.7, 100 µl of responder cells/splenocytes (1 x 10^6 cells/ml in PM) from BALB/C mice were dispensed to wells containing treated and untreated BMBDs. After 72 h in culture, the cells were pulsed with 0.4 µCi/well of [methyl-^{3}H] thymidine (Amersham Biosciences, Baie d'Urfe, PQ, Canada). After an additional 18 h of culture, cells were collected with a Filtermate harvester and thymidine uptake was measured by scintillation counting with a TopCount NXT microplate scintillation counter (Packard Instrument Company, Meriden, CT, USA). Proliferative responses were reported in counts per minute.

5.3.10 Statistical Analysis

All data were analysed with the aid of GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). For ELISA titers and ELISPOT counts, differences among groups were examined using the non-parametric Kruskal-Wallis test. If the result of an analysis of variance (ANOVA) proved significant, then multiple post-test comparisons between medians were done using a Dunn's test, or differences between the medians of two groups were examined using the Mann-Whitney U test. For *in vitro* MLR assays, differences among groups were analysed by ANOVA followed by Tukey's multiple comparisons test in case of a significant ANOVA, or differences between the means of two groups were examined by unpaired Students t-test. Differences between groups were considered significant if probability values of $p<0.05$ were obtained.

5.4 Results

5.4.1 Functional/biological activity of synthesized peptides

To verify the two synthesized forms of BNBD3 prior to their use in the peptide/DNA complexed vaccines, both synthesized peptides were assayed to ensure that they were biologically active and that the native β-defensin disulfide connectivities were achieved during oxidation (folding) of the peptides [389]. The attraction of bovine iDC to the synthesized peptides sBNBD3 and aBNBD3 was compared to that of native BNBD3 using a chemotaxis assay. Bovine iDCs migrated equally to both synthesized peptides and native BNBD3, and the data gave characteristic bell-shaped dose response curves with the same peak migration at 10 ng/ml (**Figure 5.2**). Thus both synthesized peptides were deemed suitable for use in the vaccines as they were shown to be equivalent to the native BNBD3 in chemotactic ability for iDCs, and since maximum cell migration occurred at the same concentration for all, the synthesized peptides most likely had the native conformation.

The slight changes in the amino acid sequence of sBNBD3 to that of aBNBD3 theoretically afforded aBNBD3 greater stability, and this may have accounted for its desirable ease of synthesis and greater yield when compared to that of sBNBD3. sBNBD3 has an N-terminal glutamine residue, and these residues have shown a propensity to spontaneously cyclize over time to become pyroglutamate [460]. This instability was reflected in a subsequent reduction in the yield of the purified peptide with the correct amino acid sequence, from the synthesis process. Since the conversion of glutamate to pyroglutamate has also been found to occur *in vivo* [460], this might also cause it to be unstable/unpredictable in an animal vaccine. Due to its

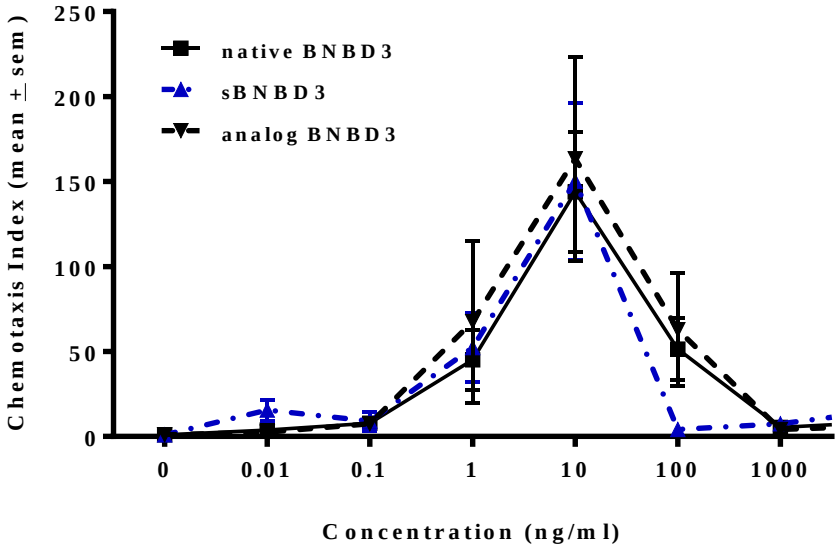

Figure 5.2 Chemotaxis of bovine iDC to native and synthesized forms of BNBD3. Bovine iDCs were labelled with Calcein AM and placed on a 5 µm pore filter membrane above triplicate bottom wells filled with either medium or 0.01, 0.1, 1, 10, 100 or 1,000 ng/ml of each BNBD3 peptide. Migration of cells toward medium or defensin was determined by reading the calcein fluorescence signal of migrated cells on the bottom of the filter after incubation at 37 °C for 90 min. Data are expressed as the chemotactic index (CI), and are shown as the mean values of the average CI ± sem of four donor animals (n=4). sBNBD3 (published sequence, Glutamine [Q] in positions 1 and 27), aBNBD3 (Glycine [G] replaces Glutamine [Q] in positions 1 and 27).

potential *in vivo* instability, and lower yield, sBNBD was deemed uneconomical and unsuitable as a vaccine component, thus only the aBNBD3-complexed vaccines were selected for evaluation.

5.4.2 Formation of peptide/DNA complexes

To characterize the complexing effect of the positively charged aBNBD3 peptide on the negatively charged pMASIA-tgD DNA vaccine, a constant amount of pMASIA-tgD (5 µg;

0.0015 nmol) was mixed with increasing amounts of aBNBD3 and analyzed by EMSA (Figure 5.3). Most of the pMASIA (Figure 5.3, lane a) and pMASIA-tgD (Figure 5.3, lane b) plasmid migrated as supercoiled DNA that moved furthest into the gel. Addition of 0.1875 nmol aBNBD3 (Medium; 125:1 nanomolar peptide to DNA ratio) reduced the electrophoretic mobility of pMASIA-tgd (Figure 5.3, lane c) and addition of 1.875 nmols, the highest amount of aBNBD (High; 1250:1 ratio), reduced the electrophoretic mobility of pMASIA-tgd to the point where the DNA did not migrate into the gel at all (Figure 5.3, lane d).

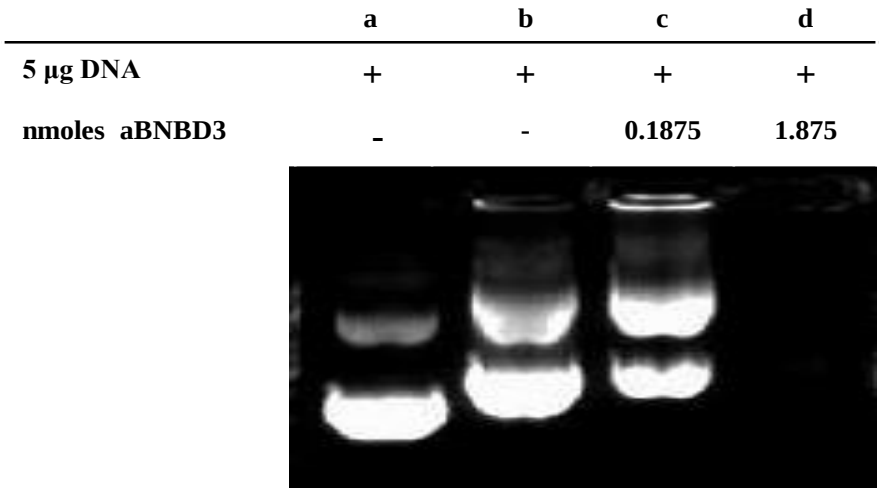

Figure 5.3 Electrophoretic mobility shift assay (EMSA) of pMASIA-tgD and aBNBD3 complexes. pMASIA-tgD DNA vaccine (5 µg) was complexed with a medium and a high ratio of aBNBD3 peptide and the DNA-aBNBD3 complexes were visualized by agarose gel electrophoresis in the presence of ethidium bromide. (a) pMASIA (placebo), (b) pMASIA-tgD, (c) pMASIA-tgD DNA complexed with 0.1875 (Medium) nmols of aBNBD3, (d) pMASIA-tgD DNA complexed with 1.8750 (High) nmols of aBNBD3. (aBNBD3; analog, Glycine [G] replaces Glutamine [Q] in positions 1 and 27).

5.4.3 Optimization of peptide/DNA ratio based on immune responses of mice to complexed vaccines

As an initial step, the optimal quantity of peptide in relation to the amount of DNA (peptide to DNA ratio) had to be established. To determine which peptide to DNA ratio would give the best results, in the first study, mice were immunized with pMASIA-tgD (5 μg; 0.0015 nmol) alone or complexed with 0.01875 (Low), 0.1875 (Medium) or 1.875 (High) nmol of aBNBD3 to give nanomolar peptide to DNA ratios of 12.5:1, 125:1 and 1250:1 respectively. The peptide concentrations were chosen based on the work of Riedl et al. (2004) [454], who showed a bell-shaped dose response curve with optimal responses to the DNA-encoded antigen at a peptide to DNA ratio of 125:1.

With the addition of aBNBD3 to pMASIA-tgD (Figure 5.4) we observed optimal humoral and unchanged CMI responses to the DNA-encoded antigen at the Medium peptide to DNA ratio of 125:1 (Figure 5.4a-d). When compared to vaccination with pMASIA-tgD alone, IgG1 (Figure 5.4a) tended to be increased, and IgG2a was significantly increased ($p<0.05$) (Figure 5.4b) by addition of the peptide at the Medium peptide to DNA ratio; whereas addition of the peptide at the high peptide to DNA ratio resulted in significantly reduced IgG2a humoral responses ($p<0.05$) (Figure 5.4b) and numbers of IFNγ secreting cells ($p<0.01$) (Figure 5.4c). There were no significant differences in the number of IFNγ (Figure 5.4c) or IL5 (Figure 5.4d) -secreting cells from mice in the pMASIA-tgD Low or Medium aBNBD3 complexed vaccine groups when

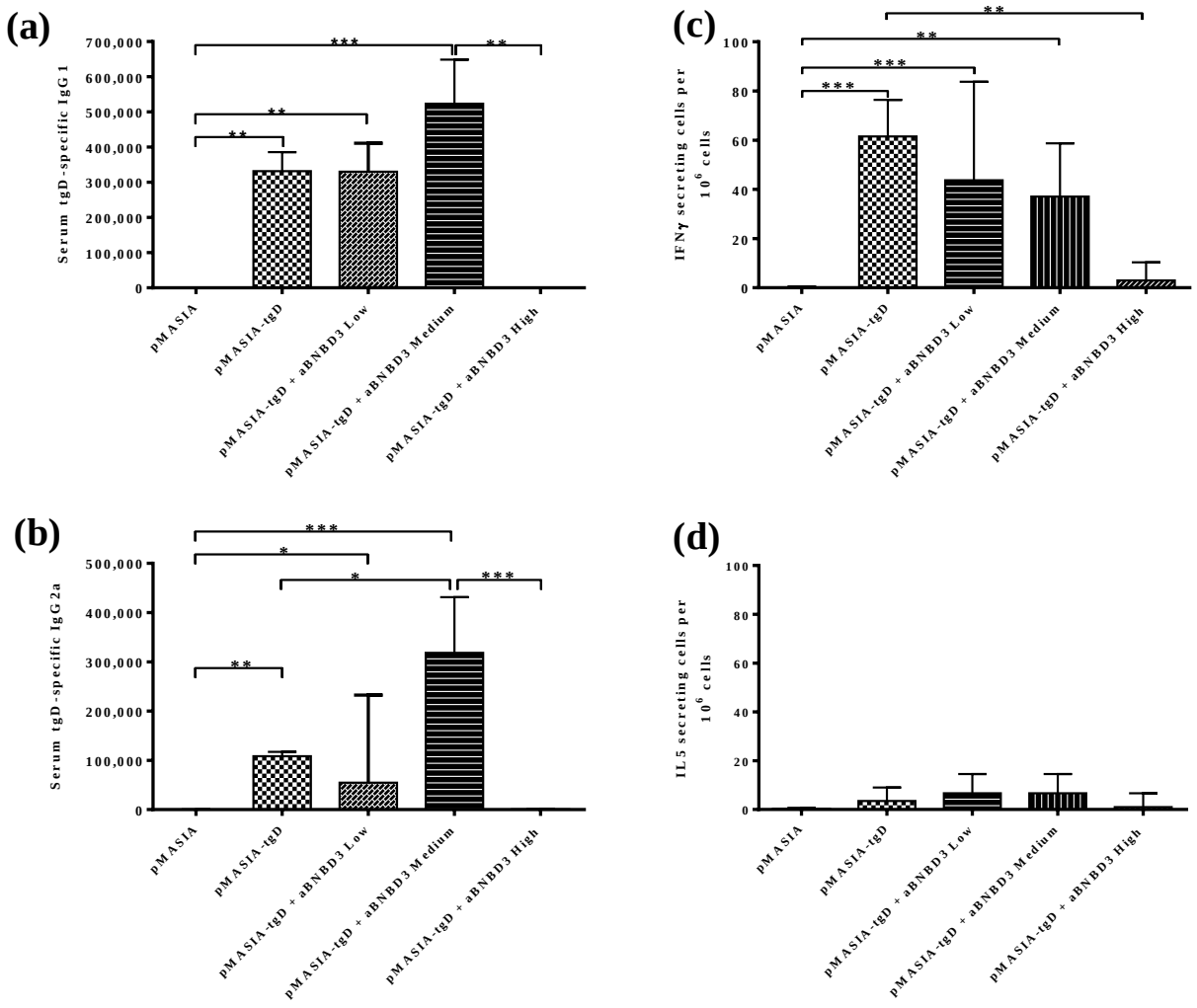

Figure 5.4 Specific (tgD) immune responses in mice immunized with aBNBD3/pMASIA-tgD complexed vaccines. C57BL/6 mice (n=8) were immunized twice ID with 5 ug pMASIA (placebo) or pMASIA-tgD DNA complexed with either 0, 0.01875 (Low), 0.1875 (Medium) or 1.8750 (High) nmols of aBNBD3 (analog, Glycine [G] replaces Glutamine [Q] in positions 1 and 27). One month after the second immunization, tgD-specific (a) IgG1, and (b) IgG2a titers were determined by ELISA. ELISA titers are expressed as the reciprocal of the highest dilution resulting in a reading of two standard deviations above the negative control. The numbers of tgD-specific (c) IFN-γ or (d) IL-5 secreting cells were measured by ELISPOT assay. ELISPOT results are expressed as the difference between the number of IFN-γ or IL-5 secreting cells in tgD-stimulated wells and medium-control wells per 10^6 cells. Bars represent the median values of each group with interquartile range. Significant differences between groups are indicated on the graphs where *p<0.05, **p<0.01 and ***p<0.001.

compared to mice vaccinated with pMASIA-tgD. These results suggested that aBNBD3 at the Medium (0.1875 nmols peptide; 125:1 ratio) nanomolar to DNA ratio, when complexed with pMASIA-tgD improved the specific antibody response to tgD encoded by the DNA vaccine while maintaining the magnitude and balance of the cellular response.

5.4.4 Immune responses in mice immunized with aBNBD3-complexed vaccines

To further explore the merits of aBNBD3/pMASIA-tgD complexed vaccine, in the second study mice were immunized with pMASIA-tgD (5 μg; 0.0015 nmol) alone or complexed with 0.1875 (Medium) or 1.875 (High) nmol of aBNBD3 to give nanomolar peptide to DNA ratios of 125:1 and 1250:1 respectively. We again observed optimal humoral and unchanged CMI responses to the DNA-encoded antigen at the Medium peptide to DNA ratio of 125:1 (Figure 5.5a-d). Specific IgG1 (Figure 5.5a) tended to be increased and IgG2a was significantly increased ($p<0.001$) (Figure 5.5b) by addition of aBNBD3 at the Medium peptide to DNA ratio when compared to the pMASIA-tgD group. There were no significant differences in the number of IFNγ (Figure 5.5c) or IL5 (Figure 5.5d) -secreting cells. Interestingly only the group vaccinated with Medium aBNBD3/pMASIA-tgD had significantly higher numbers of IFNγ-secreting cells when compared to the placebo group (pMASIA) ($p<0.05$) (Figure 5.5c). Again we observed that addition of the peptide at the High peptide to DNA ratio resulted in reduced humoral responses (Figure 5.5a-b) and numbers of IFNγ-secreting cells (Figure 5.5c) when compared to vaccination with pMASIA-tgD alone. These results are consistent with those of the first study and further confirmed that pMASIA-tgD complexed with aBNBD3 at the Medium (0.1875 nmols peptide; 125:1 ratio) nanomolar to DNA ratio improved the specific antibody response to tgD while maintaining the magnitude and balance of the cellular response.

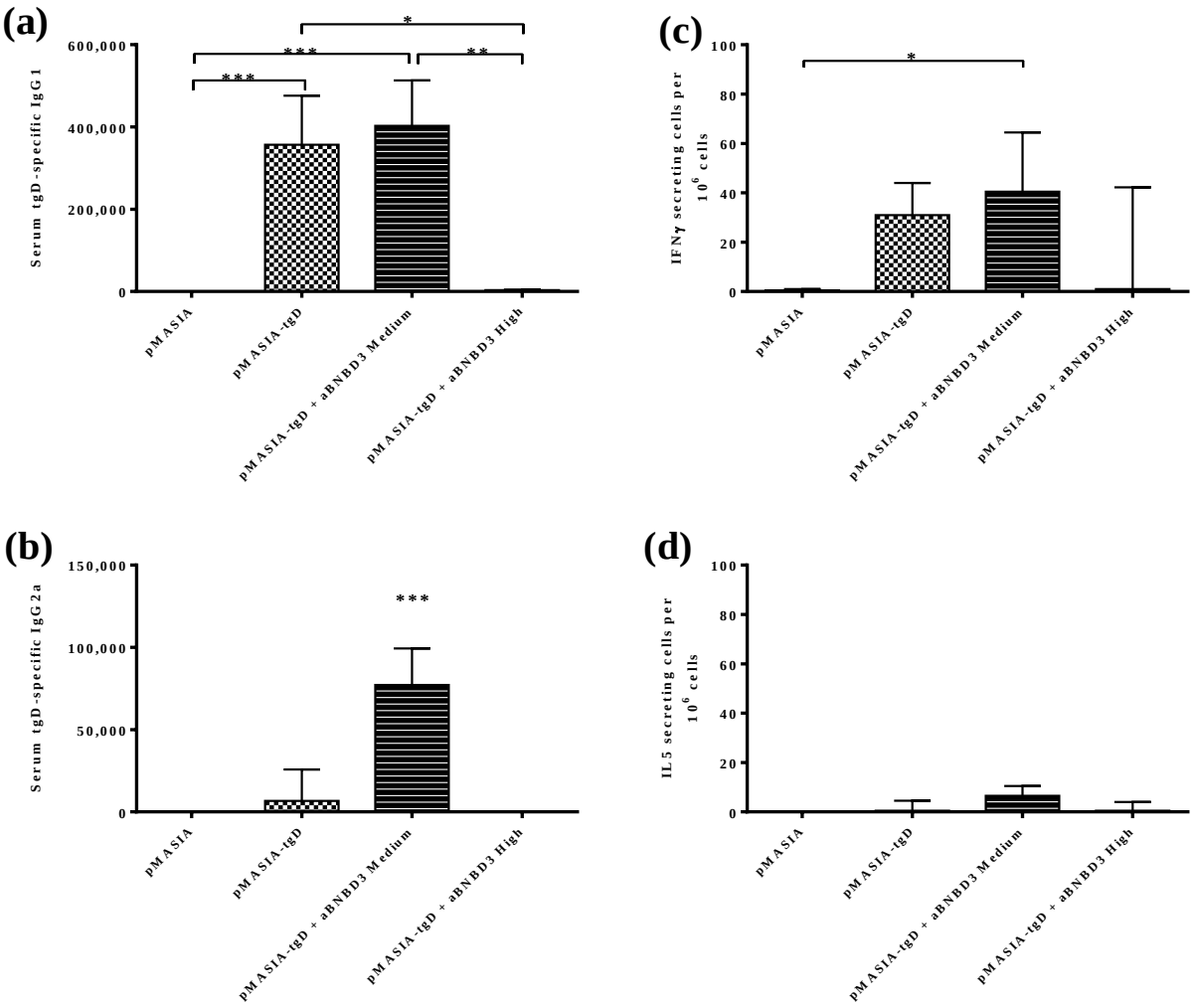

Figure 5.5 Specific (tgD) immune responses in mice immunized with aBNBD3/pMASIA-tgD complexed vaccines. C57BL/6 mice (n=8) were immunized twice ID with 5 ug pMASIA (placebo) or pMASIA-tgD DNA complexed with either 0, 0.1875 (Medium) or 1.8750 (High) nmols of aBNBD3 (analog, Glycine [G] replaces Glutamine [Q] in positions 1 and 27). One month after the second immunization, tgD-specific (a) IgG1, and (b) IgG2a titers were determined by ELISA. ELISA titers are expressed as the reciprocal of the highest dilution resulting in a reading of two standard deviations above the negative control. The numbers of tgD-specific (c) IFN-γ or (d) IL-5 secreting cells were measured by ELISPOT assay. ELISPOT results are expressed as the difference between the number of IFN-γ or IL5 secreting cells in tgD-stimulated wells and medium-control wells per 10^6 cells. Bars represent the median values of each group with interquartile range. Significant differences between groups are indicated on the graphs where *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

5.4.5 Effect of BNBD3 on maturation and activation of murine bone marrow derived dendritic cells (BMDCs)

To study the potential mechanism by which aBNBD3 increased humoral responses and modulated Th1-type responses as evidenced by induction of antibody of the IgG2a isotype, the effect of BNBD3 on maturation and functional activation of mouse BMDCs was examined. Phenotypic changes induced in BMDCs by different treatments were detected by analysis of DC markers, antigen presentation and costimulation/activation molecule expression (CD11c, MHC II, CD86, CD40) using flow cytometry (FACs). Mean fluorescent intensity (MFI) of expression, used here to measure the quantity of molecule produced in response to stimulation, was not affected by treatment with aBNBD3 for any marker with the exception of CD11c, where an increased MFI above LPS (100 ng/ml) for the aBNBD3 treated cells at 10 and 100 ng/ml concentrations was observed (Figure 5.6a). With respect to the effect on numbers of cells, treatment with LPS (100 ng/ml) or all concentrations (10, 100, 1000 ng/ml) of aBNBD3 or nBNBD3 resulted in equally high percentages (frequencies) of cells expressing CD11c (>80%) and equally moderate frequencies of cells expressing MHC II (>30%) (data not shown). Treatment with LPS (100 ng/ml), but not aBNBD3 or nBNBD3 at any concentration, resulted in strong upregulation of CD40 and CD86 costimulation/activation molecules based on a 5-10 fold increased percentage of cells expressing these molecules (data not shown).

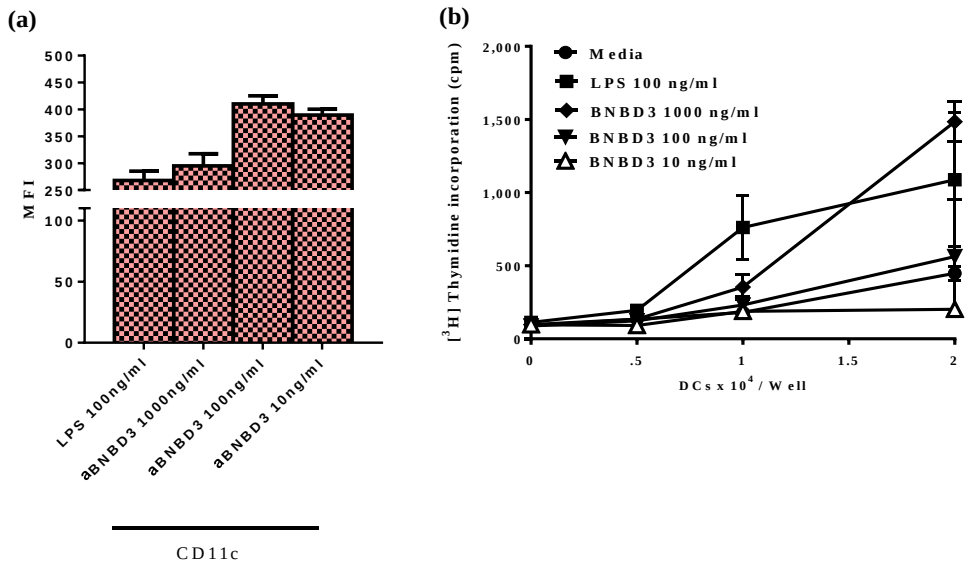

Figure 5.6 Phenotypic and proliferative changes effected by aBNBD3 treatment of Day 9 Mouse BMDCs. Day 9 BMDCs from C57BL/6 mice (n=3) were cultured for 24 hr in 6-well plates in the presence of aBNBD3 or LPS and cells were stained for expression of CD11c, CD40, CD86 and MHCII. Cells were harvested for use in the assays on day 10. (a) Treatment of BMDC with aBNBD3 at 10, 100 or 1000 ng/ml or with LPS at 100 ng/ml increased the fluorescent intensity of expression of CD11c. Data are the mean MFI (mean fluorescent intensity) $\pm$ sem of 1 well of cells from each of three mice. (b) Treatment of BMDC with aBNBD3 at 100 or 1000 ng/ml or with LPS at 100 ng/ml increased proliferation of mismatched splenocytes in an allogeneic MLR. Increasing numbers of BMDCs were incubated with 2 x 10^5 responder cells/well (BALB/C splenocytes) for 3 days. Proliferative responses were measured by the incorporation of 0.4 μCi/well [methyl-^{3}H] thymidine for 18 hr. Data are mean CPMs $\pm$ sem of triplicate wells. Similar results were obtained in a second independent experiment with nBNBD3.

Functional maturation and activation as a result of treatment with BNBD3 was determined by measuring the changes in allostimulatory capacity of treated BMDCs. An increase in proliferative ability indicative of DC maturity was observed with LPS (100 ng/ml) and with increasing numbers of DCs which was expected (Figure 5.6b). aBNBD3 at 100 and 1000 ng/ml also increased proliferation; but only the higher 1000 ng/ml aBNBD3 treatment tended to induce greater proliferation than LPS (100 ng/ml) and this was only observed when DCs were 2 x 10^4

/well (Figure 5.6b). These results suggest that BMDC incubation with aBNBD3 at 1000 ng/ml and to a lesser extent, at 100 ng/ml results in functional maturation and activation as shown by their increased allostimulatory ability. Additionally, from the dose response curve we observed, as did Yu et al. (2006) [461], that a DC to responder cell ratio of 1:10 gave the best response in MLR (Figure 5.6b). Thus a 1:10 DC to responder ratio was used in subsequent studies.

To confirm our findings that aBNBD3 stimulated functional maturation and activation of BMDCs and to elucidate differences (if any) in its activity to that of nBNBD3 and sBNBD3, BMDCs were treated with nBNBD3 (Figure 5.7a), sBNBD3 (Figure 5.7b) and aBNBD3 (Figure 5.7c) at 10, 100, and 1000 ng/ml with and without LPS at 100
ng/ml. Comparable stimulation and proliferation by the DCs treated with synthesized BNBD3s indicated that aBNBD3 and sBNBD3 acted equally when compared to nBNBD3, and at concentrations of 100 ng/ml and 1000 ng/ml acted equally when compared to LPS to mature the DCs (Figure 5.7a-c). Proliferation was greatest (equal or better than LPS at 100 ng/ml) and significant relative to the medium control for: nBNBD3 at 100 ng/ml ($p<0.01$) and 1000 ng/ml ($p<0.01$) (Figure 5.7a); sBNBD3 at 100 ng/ml ($p<0.01$) and 1000 ng/ml ($p<0.01$) (Figure 5.7b) and aBNBD3 at 10 ng/ml ($p<0.01$), 100 ng/ml ($p<0.01$) and 1000 ng/ml ($p<0.01$) (Figure 5.7c). Combined stimulation by LPS and BNBD3 was tested to determine whether changes in maturation due to BNBD3 might be seen with a second

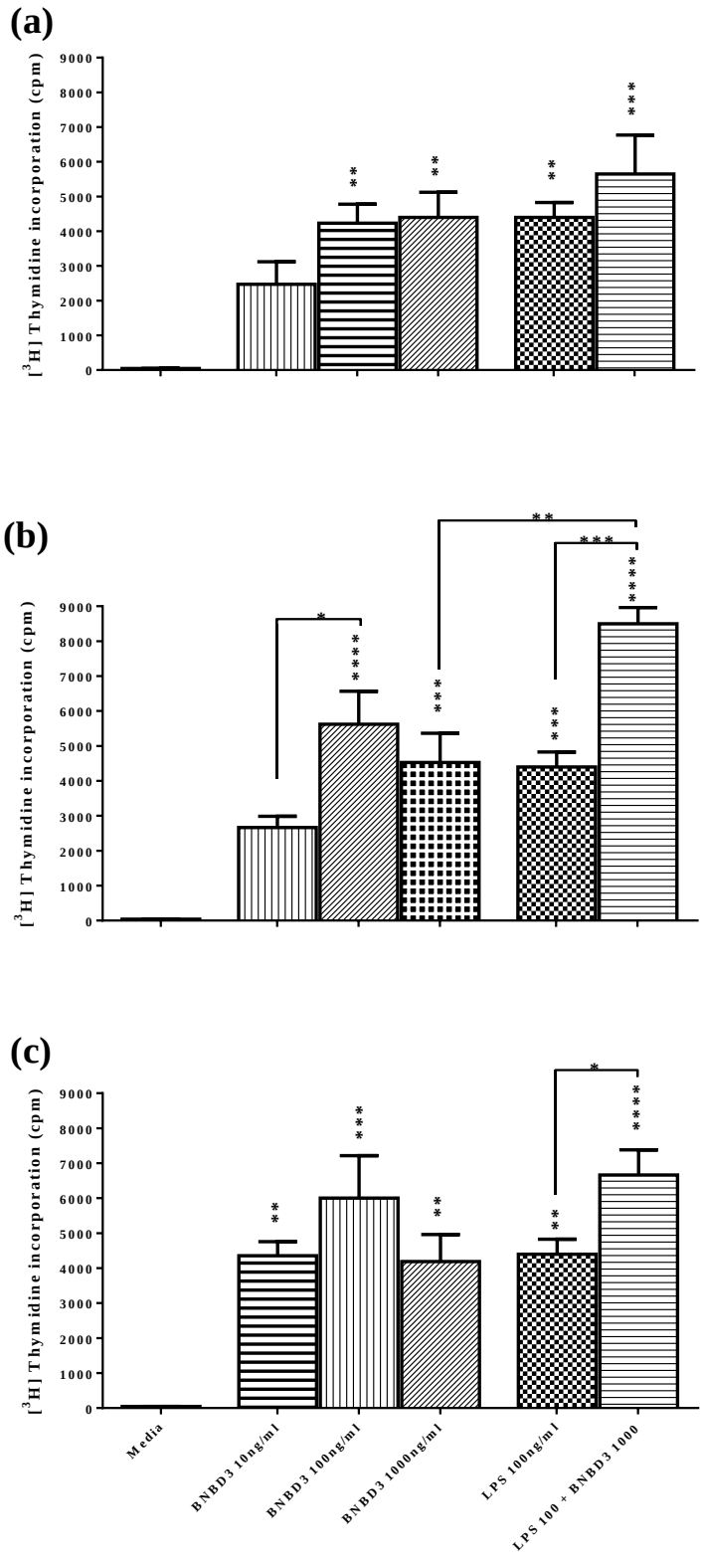
(a)
[3H] Thymidine incorporation (cpm)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
(b)
[3H] Thymidine incorporation (cpm)
(c)
[3H] Thymidine incorporation (cpm)
Media
BNBD3 10ng/ml
BNBD3 100ng/ml
BNBD3 1000ng/ml
LPS 100ng/ml
LPS 100 + BNBD3 1000

Figure 5.7 Activation/maturation measured by stimulatory ability of BMDCs in the MLR assay after treatment with nBNBD3, aBNBD3, and sBNBD3 with and without LPS. Day 5 BMDCs (1 x 10^4/well) from C56BL/6 mice (n=4) were dispensed to U-bottomed 96-well plates and treated for 18 hr with LPS (100 ng/ml), BNBD3 (10, 100, 1000 ng/ml) or LPS (100 ng/ml) + BNBD3 (1000 ng/ml). BMDCs treated with (a) native BNBD3, (b) sBNBD3 or (c) aBNBD3 were incubated with BALB/C splenocytes (1 x 10^5/well) for 3 days. Proliferative responses were measured by the incorporation of 1 μCi/well [methyl-^{3}H] thymidine for 18 hr. Data are mean CPMs ± sem of wells from each BMDC-donor mouse. Significant differences between groups are indicated on the graphs where *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

stimulation, as had been noted for other peptides [235]. The combined treatment of BNBD3 at 1000 ng/ml and LPS (100 ng/ml) tended to increase proliferation above that of LPS for all of the BNBD3s and this difference was significant for sBNBD3 ($p<0.001$) (Figure 5.7b) and aBNBD3 ($p<0.05$) (Figure 5.7c). Thus, secondary stimulation may also be involved in the activation/maturation mechanism of BNBD3 on murine BMDCs. These results confirm our previous finding that aBNBD3 induces functional maturation and activation of BMDCs. There were no differences in the proliferations induced by BMDCs treated with nBNBD3, sBNBD3 or aBNBD3.

5.5 Discussion

Control of infection with BoHV-1 by infected animals is hindered by the virus' ability to achieve latency and to employ other mechanisms that promote viral evasion of the immune system (reviewed in [59]). Induction of apoptosis of $CD4^+$ T-cells, B-cells and Mo by BoHV-1 could be expected to have a negative consequence on antibody production and on $CD4^+$ T-cell support for CTLs [462]. Viral interference with virus-specific antibodies can reduce the effectiveness of the antibody-mediated response [463]. Down-regulation of expression of MHC I would delay and

reduce detection of virus-infected cells [464, 465] and impair activation of $CD8^+$ CTL [466, 467]. Thus the ideal BoHV-1 vaccine that is effective and protective, yet also non-viral and non-infectious, is sought. Responses to the ideal BoHV-1 vaccine should be broad-based [67]; and should stimulate both cellular and humoral arms of the immune system [468].

In our quest for this "ideal" DNA vaccine we previously studied the immune response-enhancing capacity of BNBD3 [389], in a DNA vaccine as fusion construct with tgD of BoHV-1. This vaccination strategy increased cell-mediated immune responses including the induction of CTLs and was protective against BoHV-1, but these improvements did not result in enhanced clinical responses which could have been because the vaccine did not concurrently increase the humoral response [452]. We then hypothesized that the same cationic peptide, BNBD3, might improve the humoral response without loss of robust Th1-type CMI if it was formulated in a complex with pMASIA-tgD. This hypothesis was strongly influenced by the work of Reidle et al. (2004), who found that the humoral immune response to a DNA-encoded antigen could be improved, without loss of cellular responses when the DNA was complexed by a short cationic peptide at a nanomolar peptide to DNA ratio of 125:1 [453, 454]. In this current study, we showed that aBNBD3, when used at a nanomolar peptide to DNA ration of 125:1, enhanced the specific Th-1 type humoral responses of mice to tgD encoded by pMASIA-tgD, with robust Th-1 type cellular responses maintained. *In vitro* we showed that aBNBD3 exerted a direct effect on murine BMDCs; the peptide activated BMDCs and functionally matured these cells as evidenced by increased allostimulatory ability. There was no effect of amino acid substitution in aBNBD3 as comparative studies with native, sBNBD3 and aBNBD3 showed that all three peptides were equally capable of inducing functional maturation of BMDCs.

When evaluated by chemotaxis assay for structural correctness and biological activity, the synthesized BNBD3 peptides were chemotactic for bovine iDCs. Chemotactic activity was unaffected by the amino acid substitution in aBNBD3 so this peptide was used in the complexed vaccine. Although chemotactic activity for bovine iDCs does not guarantee that mouse iDC would be attracted to aBNBD3, it is likely that they would be. Chemotactic activity of hBD2 for mouse and human CCR6-expressing cells [295] and of mBD4 (the mouse orthologue of hBD2) for human CCR2-expressing cells has been demonstrated [215]. Thus, it is possible that pMASIA-tgD complexed with aBNBD3 at the medium DNA: peptide ratio of 125:1 affected an increase in the magnitude of the humoral response by inducing chemotaxis of iDCs to the site of immunization. This in turn would increase the likelihood of uptake of the DNA vaccine by iDCs followed by expression of the tgD antigen and subsequent presentation by DCs of tgD through both MHCI and MHCII pathways leading to CMI and humoral responses respectively [110, 114, 217, 218]. We have observed chemotaxis of iDCs following a bell-shaped curve in response to increasing concentrations of aBNBD3 in this and in a previous study [389]. It is possible that iDCs (and other immune cells) were optimally attracted to the concentration of aBNBD3 in the pMASIA-tgD vaccine complexed at the medium ratio of 125:1 and that this translated to a favorable improvement in the humoral response of the mice without change in the cellular response. In this scenario fewer cells would be attracted to the vaccine complex with the low concentration of peptide (12.5:1), which might explain the lower humoral and cellular response we observed. This would also explain the almost complete abrogation of *in vivo* immune responses in the group given the vaccine complexed to the highest peptide concentration (1250:1) as iDCs are so poorly chemo-attracted to high concentrations of β-defensin that they are almost repulsed. Also, where the concentration of aBNBD3 in the pMASIA-tgD vaccine

complexed at the medium nanomolar ratio of 125:1 may have made the vaccine attractive for uptake by infiltrating iDC, the high concentration of peptide complexing pMASIA-tgD at the nanomolar ratio of 1250:1 may have provided a sufficiently strong danger signal to target the vaccine for removal by extracellular processes [469], by endosomal or proteosomal degradation after cellular uptake [470] or by inducing cell death [471].

Complex formation was monitored by EMSA, and we observed a reduction or retardation, that increased as the amount of peptide added was increased, of electrophoretic mobility of the complexed DNA versus that of the naked DNA as has been described by others [472]. How complex formation of DNA with a cationic component translates to a benefit of the *in vivo* immune response is currently unknown. Increased transfection of cells by complexed DNA is one possible mechanism that would be expected to enhance the magnitude of the immune response. Others have reported however, that while higher peptide to DNA ratio complexes showed enhanced transfection efficacy *in vitro* as determined by cellular production of the DNA-encoded antigen, these same complexes resulted in suppressed immunogenicity *in vivo* [454]. Our findings are in good agreement as we also observed almost complete abrogation of both humoral and cellular responses when pMASIA-tgD was complexed at the similarly high DNA: peptide ratio of 1250:1. Of the three concentrations we tested, enhanced specific humoral responses of mice to tgD encoded by the pMASIA-tgD DNA vaccine (with cellular responses maintained) were only achieved when DNA was complexed with aBNBD3 at the medium nanomolar peptide to DNA ratio of 125:1, but not at the low or high ratio. Others have also observed this critical dependency on a particular concentration of peptide in relation to DNA, and typically the optimal amount of peptide for the *in vivo* delivered complex had to be much

lower than the amount that gave the best transfection efficiency *in vitro* [454, 473]. Interestingly, Riedl et al. (2004) also found the cationic peptide to DNA ratio that was optimal *in vivo* was 125:1 [454], thus our findings are in good agreement. Another reason for the very low response at the high ratio in vivo could be that after uptake of the complex by the cell, with increased quantity of peptide, the DNA was unable to fully release from the complex. The higher concentration of the peptide could either have hampered movement of DNA to the nucleus [473] or it could have effectively "trapped" the DNA in the endosome where ultimately the DNA would be degraded by lysosomal enzymes following merging of the lysosome with the late endosome [474]. *In vivo*, naked DNA can be degraded by serum nuclease before it is ever taken up by cells, but in a complex at the optimal ratio, aBNBD3 may have afforded protection to the DNA as has been observed for cationic liposomes [475], cationic copolymers [476] and cationic peptides such as IC30 (KLK) [477].

Due to its high molecular weight and negative charge DNA does not easily cross cell membranes [469]. Complexing with aBNBD3 may have condensed the DNA making its size and shape more attractive for cellular uptake [478, 479]. An additional mechanism may have been that aBNBD3 increased binding and entry of the DNA into cells, across the negatively charged cell membranes, as has been reported for cationic liposomes [480] and cationic peptides [477, 481]. Also, it is possible that aBNBD3 acted on iDCs as a cell-penetrating-peptide (CPP). This idea that aBNBD3 may act as a CPP is supported by the finding that many cationic CPPs are taken up with their cargo through macropinocytosis [482, 483] and the resulting macropinosomes may acidify but do not intersect with lysosomes and thus there is avoidance of lysosomal degradation [484]. It has been reported that human iDCs in the presence of hBD2 undergo actin-driven

plasma membrane ruffling and uptake of peptide suggestive of macropinocytosis with subsequent avoidance of lysosomal degradation of linked fluorophores [297]. It is possible, then, that aBNBD3 may use this same mechanism as described for hBD2 to gain entry to DCs and thus facilitate transfer of DNA into, and protection of DNA within, the cells. Following transfer, aBNBD3 might then carry the DNA as a passenger molecule into the nucleus [485], as has been described for the cationic antimicrobial peptide human LL-37 [486]. Improved trafficking into and through the cell and to the nucleus would be expected to result in an increase of the quantity of the DNA-encoded tgD protein, which in turn would have a positive effect on increasing antibody production.

When we treated BMDCs *in vitro* with aBNBD3 we observed only partial phenotypic changes suggestive of maturation relative to LPS-treatment, but the aBNBD3-treated cells that displayed the lowest CD11c (comparable to LPS) were the most capable of inducing proliferation. Although there are conflicting reports of up- or down-regulation of CD11c with maturation [487, 488], and up-regulation has been associated with increased DC survival [488], our results are in agreement with those who found down-regulation of CD11c expression to be a sign of activation and to be correlated with functional maturity [489]. Increased proliferative ability of DCs treated with aBNBD3 may provide a clue as to mechanisms for increased antibody. In analysis of allo-MLR responses, MHC I-reactive CD4 cells function as helper cells, thus increased proliferation in an allo-MLR assay can indicate induction of $CD4^+$ Th 1 cell responses [490]. Through increased CD4 Th1 cells we might expect to see increased help to B-cells that would result in increased antibody response of Th1-type. Thus, it is possible that *in vivo*, when DNA was complexed with aBNBD3 at the medium nanomolar peptide to DNA ratio of 125:1, aBNBD3

might have acted on iDCs, to increase proliferation of the $CD4^+$ Th1 subset, and through help to B-cells, increased the specific Th1-type antibody response.

In summary, we show enhanced efficacy of the humoral responses while maintaining cell-mediated responses to a DNA vaccine by the addition, at the medium nanomolar peptide to DNA ration of 125:1, of the synthesized peptide aBNBD3 as a complex with the DNA vaccine. This characteristic of the immune response was not induced previously by vaccination with the DNA vaccine fusion construct encoding BNBD3 with tgD. We show induction of IFN-γ-secreting cells and an increase in IgG2a antibody production, both of which are desirable. Since both robust antibody and CMI responses of a Th1 type are desired for protection from BoHV-1 infection, and this strategy does result in both, the results of this study support our hypothesis and indicate a direction for future studies that could be undertaken for a DNA vaccination strategy to protect cattle from challenge with BoHV-1.

6. GENERAL DISCUSSION AND CONCLUSIONS

Infectious diseases are a threat to the health and welfare of animals and humans. Worldwide, viruses that infect the respiratory tract cause the majority of human viral disease and these range in severity from a simple case of the sniffles (common cold) to that of severe pneumonia [491]. In cattle also, the most common respiratory diseases typically have a viral origin. As the causative agent of IBR and of an immune suppression that initiates BRDC in calves and feedlot cattle; as well reproductive disease causing abortion in animals of breeding age, BoHV-1 is no exception [90, 492]. The virus is considered a major pathogen in cattle and is responsible for economic loss as well as animal pain and suffering [493]. Because BoHV-1 is also able to establish lifelong latency following initial infection, the virus can be repeatedly reactivated causing recurrent infection, viral shedding, and spread of the virus [57]. Thus each animal that is infected becomes a possible reservoir or carrier for the virus. Vaccination is considered one of the best options for prevention and control of infection, but current commercial vaccines based on either MLV or KV vaccine technology platforms do not prevent infection and are either 1) safe but not sufficiently immunogenic/effective in the case of KV vaccines [494] or 2) are effective but potentially immunosuppressive, could establish infection and reactivate from latency and carry the risk of reversion to wild-type virus in the case of the MLV vaccine [382, 495]. There is a need then, for a non-infectious, yet effective, vaccine strategy that will induce both the humoral and cellular arms of the adaptive immune response. Ideally, this strategy would provide a vaccine that would prevent infection; that is safe, does not lead to an infectious state, does not induce a carrier state, does not induce pathology, that imparts long-lasting protection and that is economical and transferable. Such a vaccine strategy would not only apply to BoHV-

1, but could also extend to human viruses that establish latency such as HSV-1 and -2, as well as for high-risk viruses such as HIV, Ebola or HCV. Besides KV, current options for non-infectious vaccines include subunit (epitope- or protein-based) vaccines and DNA vaccines. Of the two, DNA vaccines mimic most closely the conditions of natural infection in that the DNA-encoded antigen(s) are produced by the host cell machinery, expressed within the cell and can be presented by MHC I, or are secreted and presented by MHC II or available for B-cell stimulation. DNA vaccines have great potential in their ability to generate both cellular and humoral immune responses [241]. The problem with DNA vaccines however, is that although they have inherent immunostimulatory or adjuvanting properties [496], induction of immune responses is slow particularly when compared to responses induced during natural viral infection [497]. In primates and large animals immune responses to DNA vaccines are often not of a magnitude sufficient to give protection from disease [252]. Many reasons have been given with subsequent investigations of various means to improve immune responses to DNA vaccines (reviewed in [498]). One most interesting strategy to improve immune responses to DNA vaccines is based on the central tenet that induction of immune responses to any pathogen or vaccine requires antigen to be presented by competent DCs [114]. Consequently, targeting vaccines to DCs or increasing the number of DCs at a vaccination site seems a logical approach to improve immune responses to DNA vaccines [135]. This approach was effective in a mouse model when a murine β-defensin, chemotactic for iDC, was able to improve the immune responses to DNA vaccines encoding otherwise non-immunogenic HIV or tumor antigen [217, 218]. Based on the findings in mice, the work in this dissertation was undertaken to test my hypothesis that a bovine β-defensin, chemotactic for bovine iDCs, could act similarly to improve

the immune responses of cattle to DNA vaccination and ultimately afford protection from BoHV-1 infection.

The first objective of this project was to assess the chemotactic activity of bovine β-defensins for bovine iDCs and select the most chemotactic (chapter 3). In order to do this, bovine DC had to be generated, and their maturation state characterized. Additionally, the bovine β-defensins had to be synthesized, validated and then screened for chemotactic activity using the defined immature bovine DCs. At the start of this project, there were few publications of bovine DCs, and in none of them had any differentiation, phenotypically or functionally, been made between the bovine immature or mature DC [309, 499-501]. Additionally there were no reports of immunological effects, including chemotaxis, for the sixteen known bovine β-defensins. Thus both parts of this study were novel in nature.

Using phenotype and functional assays I found that bovine Mo differentiated to iDCs within as few as three days (DC3) and when compared to the more conventional six-day DC cultures or DCs cultured for ten days with and without maturation factors these DC3s were at the most immature stage (Chapter 3). This protocol was the first to show that bovine iDC could be generated in less than 5-6 days. The short culture is advantageous as it gives the greatest yield at the most immature stage of DCs, should help to prevent errors due to assaying with the more "matured" day 5-6 DC, and has an added benefit in that more studies can be conducted in a shorter time. Next the sixteen known β-defensins were synthesized and fourteen were correct (BNBDs1-9, 12, 13, EBD, TAP, LAP). These were subsequently screened for chemotactic activity by the bovine iDCs (DC3) and DC6. Bovine iDC were overall more chemo-attracted to

the bovine β-defensins than were DC6, and these results were in good agreement with those from the studies of mouse defensins [214, 217]. BNBD3, BNBD9 and EBD had the greatest chemotactic activity of the fourteen synthesized peptides for iDCs. Since the activity was equal for these three, but only one peptide was to be used for the rest of the project, BNBD3 was chosen based on its relative ease of synthesis and abundance in the bovine neutrophil, and was subsequently subjected to further study.

Concerns that were raised regarding the correct folding and folding-related functionality of the synthesized peptides were addressed by synthesizing three variants of BNBD3 and comparing them with the native peptide using comparative HPLC and iDC chemotaxis. Of the three synthesized variants the one with the identical amino acid sequence as native BNBD3 (nBNBD3) co-eluted with nBNBD3 when analyzed by HPLC, which proved our synthesized BNBD3 was identical to nBNBD3. This verified the native disulphide connectivities in the synthesized peptide and showed that the oxidation conditions and synthesis method that I used would produce a correctly folded peptide. Additionally, since bovine iDCs migrated equally with the same bell-shaped dose response curves and peak migration at the same concentration to the three synthesized BNBD3 variants as to the nBNBD3, and this functionality is fold-related, these chemotactic activity results further verified that the BNBD3 peptides were correctly folded and by extension that all synthesized peptides used in this study were correctly folded.

Recruitment of DCs to a site of injury or infection by inflammatory molecules is the first step to induce an immune response to an offending pathogen and this is accomplished through co-ordinated and orderly movement of the appropriate cells. For example, others have shown that

the CCR2 ligand MCP-4/CCL13 recruits CCR2-expressing DC precursors from the blood. This direction is very specific as MCP-4/CCL13 expression is restricted to the basal epithelium in contact with the blood vessels. In response to the tissue environment, these cells lose CCR2 and differentiate to CCR6-expressing iDC that are attracted into the tissue by the CCR6 ligand MIP3α/CCL20. Again this is very specific as MIP3α/CCL20 is expressed by the epithelial cells located at the boundary with the external environment [298]. The co-ordinate expression of chemokine ligands in the tissue and receptors on the cells ensures the movement of specific cells from circulation to the tissue where they are needed. Understanding the effect of potential vaccine adjuvants on DC trafficking is important because the type of DC attracted ultimately dictates the type of immune response that results [369]. To determine whether migration to the BNBD3 peptides was directed or random, the possible mechanism(s) of attraction between the peptides and the iDC were investigated. Migration of iDCs to BNBD3 was shown to be chemotactic (directed) and not chemokinetic (random) by checkerboard analysis. I further showed that this chemotaxis was ligand-induced through G protein-coupled seven-transmembrane domain receptors when pre-treatment of DC3 with PTX (pertussis toxin) resulted in abrogation of migration to all BNBD3 peptides. This is in good agreement with the results from the murine studies as pre-treatment of iDC with PTX resulted in abrogation of chemotaxis to murine β-defensin, and subsequently the G protein-coupled seven-transmembrane domain receptor was identified as CCR6. Since chemokine receptors are G protein-coupled seven-transmembrane receptors and CCR6 activation was recently found to induce changes in lipid raft proteins that regulate cytoskeletal dynamics of cell migration [502] my studies suggest that at least one of the bovine iDC receptors for BNBD3 is a chemokine receptor and/or a receptor involved in cell migration. Since my overall objective was to develop a DNA vaccine that could

be effective when delivered ID, the next step was to ensure that BNBD3 could attract iDCs in the skin. When BNBD3 was injected into the skin of cattle, infiltrating iDCs were identified in much greater numbers in response to BNBD3 injection than in naïve skin or in skin injected with a control solution. This showed that BNBD3 had *in vivo* chemotactic activity for bovine iDCs. Future studies could be designed to identify the specific receptor(s) and the mechanism(s) involved in movement of bovine iDC to the bovine β-defensins as the bovine chemokine receptors are discovered and as antibodies to these receptors become available. These types of studies are particularly important since targeting antigens to some chemokine receptors on iDCs has the potential to induce the undesirable effect of autoimmunity [503] in addition to inducing beneficial immune responses. Ultimately such studies would provide valuable information about how bovine β-defensins work and their subsequent potential utility in vaccine design.

The point could be made that iDCs derived from Mo are too specific and do not adequately represent the full repertoire of DCs that might traffic to the skin and be involved in the induction, regulation and maintenance of the immune response. Although bovine DCs can be generated or isolated from a number of sources such as $CD14^+$ Mo [499] or pDCs [504, 505] from PBMCs, bone marrow [500], afferent lymph [318, 323], or LNs [504], and other methods may result in more representative heterogeneous DC populations, I chose to use DCs generated from purified CD14+ peripheral blood Mo because use of an uncontaminated (non-DC cells) and a reasonably homogenous (with respect to cell size) population of DCs was important in order to obtain valid results in the *in vitro* chemotaxis assay used for screening of the bovine β-defensins. Additionally, since I was most interested in attracting potentially infiltrating immature cells, generating cells with an immature phenotype and functionality that could be verified was an

important aspect of the study. Also, monocyte-derived DCs have the greatest phenotypic similarity to dermal DCs [506] and thus perhaps represent the most likely infiltrating iDC, the dermal iDC. Attracting dermal iDC would be beneficial to vaccine design as these DCs are involved in priming for CTL responses [507], and the generation of CD4+ T cell responses [508, 509]. Since populations of DCs going to the LN or already resident in the LN would be more likely to be made up of "matured" DCs, techniques that isolated DCs from afferent lymph or from LNs were rejected. Obtaining bone marrow typically requires euthanasia of the animal and in a large animal such as cattle this can be costly. Also, it may be difficult to obtain a population of DCs with sufficient purity using this method as additional buoyant density centrifugation to remove contaminating cells is needed, and to get a good yield the culture also needs to include Flt-3 Ligand [500], a cytokine with known stimulatory effect on DC differentiation, function and maturity in other species [510, 511], but undefined effects on cattle DCs. So for the purposes of this study, and the requirement for a pure unstimulated population of iDCs, this method of generating DCs from bone marrow was not used. Once the different populations generated from bovine bone marrow are identified and their stage of maturity/activation ascertained, future studies could be designed to compare chemotaxis to BNBD3 and perhaps the other bovine β-defensins of monocyte-derived iDC versus immature bone marrow-derived DCs. Additionally it may prove interesting to compare chemotaxis of mouse or human iDCs to determine whether migration to the bovine β-defensins is also species-specific or whether one or more of the bovine peptides could be candidates for inclusion in vaccines for other species.

Having established that BNBD3 was chemotactic for bovine iDCs, the next objective of this project was to construct plasmids that express BNBD3. Following successful construction of the

plasmids that express BNBD3 on a separate plasmid (pMASIA-BNBD3) or as a fusion construct with the BoHV-1 antigen tgD (pMASIA-BNBD3-tgD), I tested the effect of ID delivered BNBD3-encoding plasmids on immune responses of mice and cattle (chapter 4). An increase in the number of IFN-γ-secreting cells was seen in mice in response to BNBD3 delivered on a separate plasmid and in cattle when BNBD3 was delivered in the fusion construct. Additionally, increased proliferation indicative of $CD4^+$ T cell induction was observed in the BNBD3 fusion-vaccinated cattle. The reason for a response in mice but a lack of response with unlinked BNBD3 in cattle is unclear although this phenomenon has been observed by others [420, 421], and suggests that both the BNBD3 and the tgD genes should be expressed in the vicinity of each other in the LN. Proximity of expression of adjuvant and antigen may be an even more important consideration when the vaccine is to be delivered ID. In an earlier study, improved immune responses to a gB DNA vaccine were observed in cattle as a result of co-administration of an IL-12 encoding plasmid following gene-gun or IM delivery but not ID delivery [512]. The question of whether proximity of expression is best accomplished through coexpression by the same cell (that is virtually guaranteed by a fusion construct) or whether expression by individual cells in close proximity to each other in the LN (co-administration of separate plasmids for adjuvant and antigen) is sufficient, has not be answered definitively. Every study seems to offer up conflicting results and therefore it can't be discounted that the encoded adjuvants and antigens themselves may be having some effect. In support of the idea that adjuvant and antigen need to be linked, it was recently shown that co-administration of two vectors encoding distinct genes resulted in less than 40% coexpression of both genes by LN cells and this lack of coordinate expression translated to a failure of the vaccine to induce antitumor effect [513]. In cattle their size relative to the mouse could have resulted in greater time and distance separation of the two plasmids and

this would have made it less likely for the plasmids encoding BNBD3 and tgD to arrive and both be expressed in close proximity and in the same LN. Our data showing no effect by separate plasmids but increased proliferation and numbers of IFNγ-secreting cells in cattle vaccinated with BNBD3 as a fusion construct favors the concept that both genes should be expressed by the same cell in order to influence T cell responses. Confounding this is the possibility that the tgD antigen itself may be exerting some effect on immune responses as this has been shown in studies with gD of HSV binding to and down-regulating a cellular receptor (HVEM) whose ligation can inhibit T cell function [514]. Therefore investigation of effects of tgD represents an area for future study. Experiments could be designed to delineate effect of antigen by comparing the immune responses of cattle to a mix of two plasmids encoding BNBD3 and tgD, with those induced by BNBD3 and a different antigen(s). Since it is simpler and more multipurpose for vaccine design with other antigens if the BNBD3 can be delivered on a separate plasmid, knowing the reason for the lack of result in a large animal would be a worthwhile undertaking. To further characterize the cellular response, I investigated whether BNBD3 could influence induction of CTLs. Greater numbers of CD8+/IFNγ+ CTLs were discovered in mice and in cattle due to vaccination with BNBD3 fused to tgD. This finding provides additional support that in order to influence T cell responses, coordinate expression of adjuvant and antigen by the same cell is best. My finding that BNBD3 increased the numbers of CTLs in mice is in good agreement with the current literature that reports a positive effect on CTL responses due to inclusion of β-defensin in a mouse vaccine [218, 515]. In cattle however, CTL induction was only evaluated after and not before BoHV-1 challenge. It has been reported that antigen targeted to DC receptors such as DEC-205 is cross presented but does not prime [516]. Hence DC-targeted antigen can expand virus-specific $CD8^+$ T cell responses through cross-presentation to

already primed $CD8^+$ T cells but can't prime for $CD8^+$ CTL [517]. It is unlikely that the BNBD3 fusion vaccine did not prime CTL responses in cattle given that I saw increased CTLs in mice without infection. However since priming of CTL T cell responses is an important consideration in vaccine design for viruses and intracellular bacteria, it might be helpful to determine whether bovine CTLs arose due to the effect of BNBD3 or whether bovine CTLs arose by priming through a DC receptor. Future studies could be done to determine if CTLs would be induced without BoHV-1 infection.

Another interesting result with respect to cellular responses was observed in mice when tgD-restimulated splenocytes were evaluated by FACs analysis for surface expression of CD3, CD8 and concurrent intracellular expression of IFN-γ. I found that vaccination with the BNBD3 fusion (pMASIA-BNBD3-tgD) induced a population of cells that were negative for CD3 and thus were not T cells, but that nevertheless were positive for CD8 and IFN-γ ($CD3^-$ $CD8^+$ IFN-γ^+). Since the antibody used in these studies to identify the $CD8^+$ population stains specifically for CD8α, and DCs are currently the only known non-T $CD8\alpha^+$ cells in the mouse spleen, it is possible that the $CD3^-$ $CD8^+$ IFN-γ^+ cells observed were splenic DCs [419]. As I discussed in Chapter 4, since $CD8\alpha^+$ DCs preferentially prime CTLs, if the cells I discovered were splenic DCs, this might explain the increased CTL response. Additionally, while the IgG levels were not significantly changed by vaccination with the fusion construct, there was a trend in both mice and cattle towards a lower antibody response. This might be explained by the effect of these $CD8\alpha^+$ DCs on CD4 T cells as these cells induce apoptosis and fail to provide appropriate signals to secondary $CD4^+$ Th cells [518]. Since $CD4^+$ Th cell help to B cells is critical for development of a robust antibody response [519, 520], such an effect on $CD4^+$ Th cells could

ultimately lead to a weak or lowered antibody response. The existence of a $CD3^{-}$ $CD8^{+}$ IFN-γ^{+} cell population as a result of DNA immunization with β-defensin-antigen fusion constructs has not been reported previously. Given the potential for $CD8\alpha^{+}$ DC cells to influence both the cellular and humoral responses further studies could be designed to positively identify this population of interesting $CD3^{-}$ $CD8^{+}$ IFN-γ^{+} cells to determine if they are $CD8\alpha^{+}$ splenic DCs.

To evaluate the effect of BNBD3 on the protective responses, cattle were then challenged with BoHV-1. The addition of BNBD3 to tgD as a fusion construct increased the magnitude of the IFN-γ response, induced tgD-specific $CD8^{+}$ T cells, and increased the proportion of CTLs in the $CD8^{+}$ T cell subset, but this improvement in cellular responses did not translate into improved protection (reduction in virus shedding, rectal temperature and weight loss) that I would have expected. Instead, cattle were protected equally by both tgD-encoding DNA vaccines. In looking for an explanation, it became apparent that since BNBD3 was unable to improve the humoral response and insufficient humoral immune responses can be a characteristic of DNA vaccination [521], and have been implicated in a lack of protection from BoHV-1 challenge, that antibody production may have been too low. In addition to the most important method of protection against BoHV-1 which is that of neutralizing the virus or blocking virus entry into cells, other antibody related mechanisms may also be important for early control of virus infectivity. Antibody-dependent uptake of virus by pDCs (plasmacytoid DCs) has been shown to be important in control of foot-and-mouth disease virus (FMDV). FMDV inhibits induction of antiviral molecules particularly type I interferons alpha/beta (IFNs α/β) as an escape mechanism. Virus alone stimulated little if any type I IFN, but incubations with virus-immune complexes (antibody coated virus) induced large quantities of type I IFN from pDCs [504]. Thus induction

of virus-specific antibody with subsequent triggering of IFNs α/β release by pDCs was critically important to the overcoming the subduing effect of the virus and to regaining the early antiviral response and thus early control of FMDV infection. It is not currently known whether antibody dependent uptake of BoHV-1 by pDCs with subsequent release of IFNs α/β occurs in BoHV-1 infection, but since herpesviruses such as HSV-1 [522, 523] and BoHV-1 [524] have also devised ways to evade the antiviral effects of type I IFNs, it is sensible to think that the innate immune systems of cattle may use this same method to mount an antiviral IFN response to many viral infections. Thus in theory, improving antibody production induction as a result of vaccination could potentially increase antibody-dependent uptake of virus by pDCs with subsequent release of IFNs α/β and this might result in better early control of BoHV-1 infection by challenged animals. Interestingly, we often speak of the influence of the innate response on the subsequent development of the adaptive response, but this is an example of the reverse; how well-developed adaptive responses can influence induction of innate responses.

There have been reports of slow, low magnitude humoral responses to DNA vaccines encoding the gD protein [311]. In this study I similarly observed a trend towards low antibody response. More importantly, I did not observe an improvement of humoral responses in mice or cattle regardless of whether BNBD3 was delivered separately or as a fusion. Since induction of naïve B cells to antigen-specific B cells is a first step in the development of humoral response, and this first step as well as amplification of antibody production by antigen-specific B cells requires sufficient good quality free or soluble antigen, the production/availability of such antigen may be a weakness of DNA vaccines in general and one that the addition of BNBD3 was unable to overcome/improve. The importance of sufficient soluble antigen in humoral responses to DNA

vaccine can be observed in the improvement seen in prime-boost vaccine regimens where free protein antigen is provided in the boost [525]. There are a number of ways in which tgD production by the transfected host cells may have been affected in this study. In chapter four, I show in a schematic overview how the plasmids were designed such that the coding sequence for BNBD3 could be inserted in three pieces either without or with a linker using the same BamHI and HindIII restriction sites for insertion into both pMASIA and the constructed pMASIA-tgD. Using this method I was able to reduce any potential for variability of expression due to non-coding differences in the plasmids. Although there was a risk of negative effect on tgD expression by another molecule being encoded N-terminally, I decided to place the BNBD3 in front of tgD based on good results, particularly with respect to generation of neutralizing antibody that requires antigen to be in the correct structurally folded form, from mouse studies with placement of mBD2 in an N-terminal position to the antigen [217, 218]. I similarly separated BNBD3 from the antigen using a well-characterized octapeptide linker to minimize any potential effect of the peptide on tgD structure. Since *in vitro* expression of tgD from the constructed pMASIA-tgD and from pMASIA-BNBD3-tgD was excellent when compared to the standard plasmid I concluded that neither the construction of pMASIA-tgD nor the N-terminal addition of BNBD3 could have had a negative impact on tgD expression that would account for the relative lack of humoral response to vaccination. The *in vivo* impact of N-terminal BNBD3 on specific tgD responses could be investigated in the future. Such a study would involve construction of a plasmid with the coding sequence for BNBD3 inserted at the carboxyl end of tgD and comparison with the plasmid that encodes BNBD3 N-terminally to tgD on 1) *in vitro* binding of the expressed tgD to mAbs specific for a number of structural neutralizing epitopes [526], and 2) immune responses in mice and/or cattle.

Another way in which tgD production by the transfected host cells may have been limited by BNBD3 is related to the potential for damaging effect of β-defensin on the inside of the host cell. HNP1, a human α-defensin, induced apoptosis of cells transfected with plasmid expressing the peptide. This was determined to be due to the effect of accumulation of HNP1 inside the cells and not from extracellular HNP1. In their study the authors constructed their plasmid to encode the mature or active form of the HNP1 peptide [527] that would be equivalent to our BNBD3. Although HNPs and BNBDs can be safely stored in the neutrophil this is thought to be due in part to the presence of a protective anionic propiece, and when this propiece is removed by proteolytic cleavage, the resulting peptide in its "mature" form is active and has the potential to be cytotoxic. Most of the effect of HDPs including the β-defensins has focused on the cytotoxic specificity of extracellular HDPs particularly in cancer research due to their specificity for negatively charged membrane components which differ between cancer and non-cancer cells [528]. Consequently, little is known of the effects of intracellular expression on DCs or on other populations of resident cells in skin, muscle or lymphoid tissue that one would expect to transfect with a DNA vaccine. In another study human aMΦ were transfected with mRNA encoding hBD2. After 24 h treatment, cell viability was reduced due to transfection with the hBD2 mRNA. In longer cultures, sadly only aMΦ cells infected with *M. tuberculosis*, which would have given the hBD2 an intracellular target and effectively distracted the peptide from targeting other structures inside the cell, were studied. Even so, after 2 days, the controlling effect of hBD2 on bacterial growth was lessened which may have been due to cytotoxic effect of the peptide [529]. Delineation of intracellular cytotoxicity of BNBD3 for DCs and other APCs, or for populations of resident cells in skin, muscle or lymphoid tissue could be a very interesting area for future

study. This knowledge would have a direct impact on determination of the value of BNBD3 or other similar peptides in subsequent DNA vaccination strategies.

In chapter five, as the final objective of this project, I investigated whether humoral responses could be improved without loss of CMI by utilizing BNBD3 in its peptide form as a complex with the DNA vaccine. The observation that BNBD3 when encoded in the DNA as a fusion construct was able to improve cellular responses (particularly with respect to CTL), yet was unable to improve the humoral response had led me to investigate whether BNBD3 could be utilized in other ways. After review of the literature I came across some intriguing reports that when a small cationic peptide fused to a short antigenic epitope was complexed at a low peptide to DNA ratio of 125:1 with a DNA vaccine encoding for a full length antigen, the humoral immune response to the DNA-encoded antigen could be improved without loss of CMI responses. This represented an ideal in terms of response to vaccination, and since this strategy had not been attempted using a cationic peptide such as a β-defensin, I decided to try using the BNBD3 peptide in this novel way. In mice, the vaccine formulated with aBNBD3 (analog) at the nanomolar peptide to DNA ratio of 125:1 tended to increase IgG1 and significantly increased IgG2a humoral responses relative to pMASIA-tgD. There was no difference in the number of IFN-γ- and IL-5-secreting cells observed in mice in response to this BNBD3-complexed vaccine compared to pMASIA-tgD, thus using this strategy I was able to maintain the size and balance of the cellular response. The finding of increased antibody of the IgG2a isotype with no increase in the number of IL-5^{+} cells suggested that BNBD3, used in this manner, might also have strengthened the Th1-type response. Contrasting these positive results, I observed almost complete abrogation of both humoral and cellular responses when a 10X greater quantity of

BNBD3 (1250:1) was used. Our results agreed with the findings from the earlier study by Riedl et al. (2004) [454], and gave rise to two questions: 1) how might BNBD3 peptide influence the humoral response, and 2) why would responses be improved at one concentration and yet be almost completely abrogated or suppressed at another? With regard to addressing these questions, through *in vitro* investigations of effects of exogenous BNBD3 on BMDCs, I observed that when mBMDCs were treated with nBNBD3, sBNBD3 or aBNBD3 the cells acquired greater stimulatory ability indicative of functional maturation/activation. This is a desirable effect for a vaccine adjuvant and additionally is in good agreement with the findings from the mouse studies on the maturing effects of mouse β-defensins on mBMDCs [216]. Of relevance to our results, by increasing the proliferative ability of DCs, aBNBD3 may have increased the antibody response. As discussed in chapter 5, proliferation can be used as a measure for induction of $CD4^+$ Th cells. Since $CD4^+$ Th cells provide help to B-cells, an increase in these $CD4^+$ Th cells theoretically could lead to increased antibody production by B cells [530]. Further *in vitro* studies to determine whether aBNBD3-treated mBMDCs increase induction of $CD4^+$ Th cells and whether these cells are primarily Th1 or Th2 could be done to address whether this is a potential mechanism for improved antibody induction.

Another possibility for how the complexed vaccine may have affected the humoral response could be from the action of aBNBD3 on the iDCs with subsequent effect on the NK cell subset [531], or from the action of aBNBD3 directly on the natural killer (NK) cell subset [532]. With DNA complexed to aBNBD3 at the medium nanomolar peptide to DNA ratio of 125:1 the vaccine *in vivo* may have improved the IgG2a response by increasing the number or activity of natural killer (NK) cells. Studies have shown that TLR-stimulated DCs increase NK cell activity

[533]. Interestingly, the mouse dendritic cell marker CD11c is down-regulated upon DC activation through TLR triggering [489]. It is provocative then, given our results whereby treatment of BMDC with aBNBD3 led to maturation and down-regulated CD11c expression, to think that aBNBD3 may have similarly triggered TLRs to activate/stimulate DCs which in turn acted on the NK cell subset to increase their numbers and activity. Since there is considerable crosstalk between NK cells and DCs or macrophages, it could be difficult to separate whether aBNBD3 may have acted directly on the NK cells; or whether it may have acted by first activating/stimulating DCs with the stimulated DCs subsequently exerting their influence on the NK cells; or both. Whether NK cells can be directly stimulated or whether accessory cells or cytokines are required for NK cell activation is currently a matter of some controversy [534]. Regardless, since activation of NK cells has been shown to increase antigen specific IgG2a responses [535], the increased IgG2a that we observed might have occurred as a result of aBNBD3-induced activity of NK cells either by direct effect on the NK cells or indirectly through the effect of aBNBD3-activated DCs.

I also studied *in vitro* chemotactic activity of BNBD3 and showed that all forms of BNBD3 were chemotactic for bovine DCs. Thus chemoattraction to aBNBD3 could have played a role in bringing iDCs to the vaccination site and this may have increased the likelihood that iDCs would take up the DNA vaccine. Since iDCs can uniquely utilize a direct phagosome to cytosol pathway [109], theoretically uptake by iDCs should result in a greater number of plasmids circumventing the destructive elements of the endosome/lysosome, thus increasing the opportunity for plasmids to reach the nucleus and subsequently resulting in higher expression of the tgD antigen. Increased tgD expression through improved chemotaxis and uptake of

pMASIA-tgD by iDCs is one way that BNBD3-complexed vaccine may have increased the antibody response in a concentration-dependent manner. I observed that *in vitro* chemotaxis by iDCs followed a bell-shaped curve in response to increased concentration with substantially lower chemotaxis by iDCs at the highest concentration of BNBD3. *In vivo* antibody responses followed a similar bell-shaped curve in response to increased concentration/amount of BNBD3 in the complexed vaccine. If, *in vivo,* fewer iDCs were attracted to the higher amount of BNBD3, and this resulted in less migration of iDC, there would be less opportunity for uptake of pMASIA-tgD by iDC. Thus, low numbers of infiltrating DCs would encourage increased degradation of plasmid in extracellular spaces and transfected somatic cells, which would translate to subsequently poor expression of tgD. This poor expression of tgD could account for the low *in vivo* humoral responses to the vaccine complexed with the high amount of BNBD3. The potential relationship between iDC chemotaxis to BNBD3 and tgD expression could be confirmed by future studies of *in vitro* chemotaxis by murine BMDCs to BNBD3 and to the complexed vaccines. Additionally, studies of uptake and subsequent expression in cell lines versus murine BMDCs of the BNBD3 complexed vaccines might clarify whether the effect of the vaccine was through increased expression of tgD by DCs (an effect that may not be observed when using cells from cell lines), and additionally elucidate the effect of concentration of the peptide on subsequent expression of the DNA-encoded antigen.

Although I did not do investigations to determine receptor(s) for BNBD3, my results suggest a mechanism(s) for activity by BNBD3 which may be receptor-mediated and this represents an area that would be interesting for future studies. There are at least 40 receptors expressed by iDCs that function primarily in antigen capture [536]. CD205 is a type I C-type lectin containing

multiple calcium-dependent binding domains and a unique cytoplasmic tail. CD205 may direct captured antigens to specialized antigen-processing compartments [134]. Three type II C-type lectins that are important for receptor-mediated antigen uptake include: DC immunoreceptor (DCIR) [537], DC-associated C-type lectin-2 (dectin-2) [538, 539], and C-type lectin receptor 1 (CLEC-1) [540]. DCIR contains a cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) and can bind glycosylated ligands. Ligands for dectin-2 and CLEC-1 have not been identified as of yet. The MMR and Fc receptors FcγR and FcεR, are also involved in antigen handling by iDCs [132, 541-544]. Chemokine receptors CCR1, CCR2, CCR5, and CCR6 are expressed on iDCs [296], and they are members of the CC cytokine family of G-protein-coupled 7-transmembrane-domain receptors (GPCR). The enhanced immune response I observed after immunization with the BNBD3-tgD fusion (chapter 3) suggests an increased efficiency of antigen uptake, processing and presentation that is suggestive of chemokine binding to CC chemokine receptor.

Other chemokine receptors are expressed by iDCs that are not strictly uptake receptors but play roles in antigen uptake and in regulating DC activation and may be influenced by BNBD3. These include two members of the immunoglobulin superfamily (IgSF); Down-Regulated by Activation (DORA), a receptor that is also involved in homing and recirculation of DC and is down-regulated following CD40L engagement [545], and Immunoglobulin-like transcript 3 (ILT3) that contains an ITIM in its cytoplasmic domain [546]. ILT3 is involved in antigen uptake and processing; it can be efficiently internalized upon cross-linking and can deliver its ligand to an intracellular compartment for processing. Heat-shock protein receptors are expressed at the cell surface where they capture hsp bound to tumor, bacterial and viral antigens [547]. Some

TLRs are expressed on iDCs (ie. TLR3,4); they have many ligands including microbial lipopeptides [548]. Additionally, some of the chemokine receptors are actually intracellular proteins. These could be utilized by BNBD3 and could theoretically affect the fate of BNBD3-tgD fusion or the BNBD3: tgD complexed vaccine. The p55/fascin protein is an actin-bundling protein that is involved in migration through the organization of a specialized cytoskeleton that also promotes antigen capture [549]. Cdc42 and Rac1 are members of the Rho family of GTPases that are involved in regulating macropinocytosis and antigen processing [550-552]. The ubiquitin protein family member, di-ubiquitin, contains two ubiquitin moieties and functions to direct internalized antigens towards DC proteasomes for processing [553].

Aside from exogenous effects on chemotaxis and uptake of DNA vaccine by iDCs, BNBD3 peptide may have influenced the fate of the plasmid and hence antigen expression and subsequent antibody response by modifying intracellular processes such as release of intracellular DNA by transfected cells, stability of DNA in the cytoplasm and targeting of DNA to the nucleus. Similar studies, such as those conducted by Sandgren et al. (2004), where a biotinylated peptide and YOYO-1 fluorophore labelled DNA were used to track movement of peptide and DNA in the cells, could be done to determine whether BNBD3, like LL37 protects plasmid DNA from nuclease degradation and whether BNBD3 targets the plasmid DNA to the nucleus [486].

In summary, this dissertation describes a series of studies undertaken with the overall objective to develop a transferable and non-infectious vaccine strategy that through targeting vaccines to DCs induces both the humoral and cellular arms of the adaptive immune response effective to

prevent infection of cattle by BoHV-1. With DC chemotaxis as a central unifying theme of all three studies, I show in the first study how bovine iDC are chemoattracted to BNBD3 (chapter 3). I next show how chemoattraction by iDC to the BNBD3-tgD fusion protein expressed by transfected skin cells at the vaccination site could explain the increased numbers of $CD8^+$/ IFN-γ^+ CTLs induced by this vaccine. I also explain how the effects of BNBD3 on the inside of the cell may have limited expression of the construct and thus production of the antigen resulting in a less than robust antibody response (chapter 4). Finally in the fifth chapter I describe how the same attraction of iDC to BNBD3 in a peptide/DNA complexed vaccine could have influenced increased transfection of iDCs with the DNA vaccine and how this may have led to increased antibody production while maintaining robust CMI responses (chapter 5). The information gained from the research presented in this thesis, as well as the current and future knowledge of the modulating effects of β-defensins and indeed all cationic peptides on immune responses, is expected to ultimately lead to the development of new effective DNA vaccines.

7. REFERENCES

1. Roizman, B., Pellet, P.E. (2001) The Family Herpesviridae: A Brief Introduction. In *Fields' Virology* (D. M. Knipe, P. M. Howley and et al., eds), Lippincott, Williams & Wilkins, Philadelphia, PA 2381-2397.
2. Spear, P.G. (1993) Entry of alphaherpesviruses into cells. *Seminars in Virology* **4**, 167-180.
3. Guo, H., Shen, S., Wang, L., Deng, H. (2010) Role of tegument proteins in herpesvirus assembly and egress. *Protein Cell* **1**, 987-98.
4. Johnson, D.C., Baines, J.D. (2011) Herpesviruses remodel host membranes for virus egress. *Nat Rev Microbiol* **9**, 382-94.
5. Roizman, B., Taddeo, B. (2007) The strategy of herpes simplex virus replication and takeover of the host cell. In *Human Herpesviruses: Biology, Therapy and Immunoprophylaxis* (A. Arvin, G. Campadelli-Fiume, E. Mokarski, P. S. Moore, B. Roizman, R. Whitley and K. Yamanishi, eds), Cambridge University Press, Cambridge.
6. Zaichick, S.V., Bohannon, K.P., Smith, G.A. (2011) Alphaherpesviruses and the cytoskeleton in neuronal infections. *Viruses* **3**, 941-81.
7. Rock, D.L. (1993) The molecular basis of latent infections by alphaherpesviruses. *Seminars in Virology* **4**, 157-165.
8. Deruelle, M.J., Favoreel, H.W. (2011) Keep it in the subfamily: the conserved alphaherpesvirus US3 protein kinase. *J Gen Virol* **92**, 18-30.
9. Tobian, A.A., Quinn, T.C. (2009) Herpes simplex virus type 2 and syphilis infections with HIV: an evolving synergy in transmission and prevention. *Curr Opin HIV AIDS* **4**, 294-9.
10. Liashkovich, I., Hafezi, W., Kuhn, J.M., Oberleithner, H., Shahin, V. (2011) Nuclear delivery mechanism of herpes simplex virus type 1 genome. *J Mol Recognit* **24**, 414-21.
11. (2011) *Virus Taxonomy: Classification and Nomenclature of Viruses, Ninth Report of the International Committee on Taxonomy of Viruses.* Elsevier Academic Press, San Diego, CA.
12. Arvin, A., Gilden, D. (2013) Varicella-Zoster Virus. In *Fields' Virology* (D. M. Knipe and P. M. Howley, eds), Lippincott, Williams & Wilkins, Philadelphia, PA 2015-2057.
13. Mori, I., Nishiyama, Y. (2005) Herpes simplex virus and varicella-zoster virus: why do these human alphaherpesviruses behave so differently from one another? *Rev Med Virol* **15**, 393-406.
14. Thiry, J., Keuser, V., Muylkens, B., Meurens, F., Gogev, S., Vanderplasschen, A., Thiry, E. (2006) Ruminant alphaherpesviruses related to bovine herpesvirus 1. *Vet Res* **37**, 169-90.
15. Pellett, P.E., Roizman, B. (2007) The Family Herpesviridae: A Brief Introduction. In *Fields' Virology* (D. M. Knipe and P. M. Howley, eds), Lippincott, Williams & Wilkins, New York, NY 2479-2499.
16. Davison, A.J. (2010) Herpesvirus systematics. *Vet Microbiol* **143**, 52-69.
17. (2012) Virus taxonomy: classification and nomenclature of viruses, Ninth Report of the International Committee on Taxonomy of Viruses. (A. M. Q. King, M. J. Adams, E. B. Carstens and E. J. Lefkowitz, eds), Elsevier Academic Press, San Diego, CA.
18. Pellett, P.E., Roizman, B. (2013) Herpesviridae. In *Fields' Virology* (D. M. Knipe and P. M. Howley, eds), Lippincott, Williams & Wilkins, Philadelphia, PA 1802-1822.

19. Deregt, D., Jordan, L.T., van Drunen Littel-van den Hurk, S., Masri, S.A., Tessaro, S.V., Gilbert, S.A. (2000) Antigenic and molecular characterization of a herpesvirus isolated from a North American elk. *Am J Vet Res* **61**, 1614-8.
20. Spear, P.G., Roizman, B. (1972) Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpesvirion. *J Virol* **9**, 143-59.
21. Zhou, Z.H., Chen, D.H., Jakana, J., Rixon, F.J., Chiu, W. (1999) Visualization of tegument-capsid interactions and DNA in intact herpes simplex virus type 1 virions. *J Virol* **73**, 3210-8.
22. Furlong, D., Swift, H., Roizman, B. (1972) Arrangement of herpesvirus deoxyribonucleic acid in the core. *J Virol* **10**, 1071-4.
23. Roizman, B., Knipe, D.M., Whitley, R. (2013) Herpes Simplex Viruses. In *Fields' Virology* (D. M. Knipe and P. M. Howley, eds), Lippincott, Williams & Wilkins, Philadelphia, PA 1824-1897.
24. Liu, F., Zhou, Z.H. (2007) Comparative virion structures of human herpesviruses. In *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis.* (A. Arvin, G. Campadelli-Fiume and E. Mocarski, et al., eds), Cambridge University Press, Cambridge.
25. Klupp, B.G., Granzow, H., Karger, A., Mettenleiter, T.C. (2005) Identification, subviral localization, and functional characterization of the pseudorabies virus UL17 protein. *J Virol* **79**, 13442-53.
26. Gibson, W. (1996) Structure and assembly of the virion. *Intervirology* **39**, 389-400.
27. Liu, B., Stinski, M.F. (1992) Human cytomegalovirus contains a tegument protein that enhances transcription from promoters with upstream ATF and AP-1 cis-acting elements. *J Virol* **66**, 4434-44.
28. Everly, D.N., Jr., Feng, P., Mian, I.S., Read, G.S. (2002) mRNA degradation by the virion host shutoff (Vhs) protein of herpes simplex virus: genetic and biochemical evidence that Vhs is a nuclease. *J Virol* **76**, 8560-71.
29. Gilbert, M.J., Riddell, S.R., Plachter, B., Greenberg, P.D. (1996) Cytomegalovirus selectively blocks antigen processing and presentation of its immediate-early gene product. *Nature* **383**, 720-2.
30. Browne, E.P., Shenk, T. (2003) Human cytomegalovirus UL83-coded pp65 virion protein inhibits antiviral gene expression in infected cells. *Proc Natl Acad Sci U S A* **100**, 11439-44.
31. Krummenacher, C., Carfi, A., Eisenberg, R.J., Cohen, G.H. (2013) Entry of herpesviruses into cells: the enigma variations. *Adv Exp Med Biol* **790**, 178-95.
32. Spear, P.G., Longnecker, R. (2003) Herpesvirus entry: an update. *J Virol* **77**, 10179-85.
33. Roizman, B., Knipe, D.M. (2001) Herpes simplex viruses and their replication. In *Virology* (D. M. Knipe, P. M. Howley and E. al., eds), Lippincott, Williams & Wilkins, Philadelphia, PA 2399-2460.
34. Spear, P.G., Eisenberg, R.J., Cohen, G.H. (2000) Three classes of cell surface receptors for alphaherpesvirus entry. *Virology* **275**, 1-8.
35. Reske, A., Pollara, G., Krummenacher, C., Chain, B.M., Katz, D.R. (2007) Understanding HSV-1 entry glycoproteins. *Rev Med Virol* **17**, 205-15.
36. Geraghty, R.J., Krummenacher, C., Cohen, G.H., Eisenberg, R.J., Spear, P.G. (1998) Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. *Science* **280**, 1618-20.

37. Dasika, G.K., Letchworth, G.J., 3rd (1999) Cellular expression of bovine herpesvirus 1 gD inhibits cell-to-cell spread of two closely related viruses without blocking their primary infection. *Virology* **254**, 24-36.
38. Gross, K.L., Porco, T.C., Grant, R.M. (2004) HIV-1 superinfection and viral diversity. *AIDS* **18**, 1513-20.
39. Fultz, P.N. (2004) HIV-1 superinfections: omens for vaccine efficacy? *AIDS* **18**, 115-9.
40. Smith, D.M., Richman, D.D., Little, S.J. (2005) HIV superinfection. *J Infect Dis* **192**, 438-44.
41. Meurens, F., Schynts, F., Keil, G.M., Muylkens, B., Vanderplasschen, A., Gallego, P., Thiry, E. (2004) Superinfection prevents recombination of the alphaherpesvirus bovine herpesvirus 1. *J Virol* **78**, 3872-9.
42. Meurens, F., Keil, G.M., Muylkens, B., Gogev, S., Schynts, F., Negro, S., Wiggers, L., Thiry, E. (2004) Interspecific recombination between two ruminant alphaherpesviruses, bovine herpesviruses 1 and 5. *J Virol* **78**, 9828-36.
43. Dasika, G.K., Letchworth, G.J. (2000) Homologous and heterologous interference requires bovine herpesvirus-1 glycoprotein D at the cell surface during virus entry. *J Gen Virol* **81**, 1041-9.
44. van Drunen Littel-van den Hurk, S., Braun, R.P., Lewis, P.J., Karvonen, B.C., Baca-Estrada, M.E., Snider, M., McCartney, D., Watts, T., Babiuk, L.A. (1998) Intradermal immunization with a bovine herpesvirus-1 DNA vaccine induces protective immunity in cattle. *J Gen Virol* **79 (Pt 4)**, 831-9.
45. Ligas, M.W., Johnson, D.C. (1988) A herpes simplex virus mutant in which glycoprotein D sequences are replaced by beta-galactosidase sequences binds to but is unable to penetrate into cells. *J Virol* **62**, 1486-94.
46. Hanon, E., Keil, G., van Drunen Littel-van den Hurk, S., Griebel, P., Vanderplasschen, A., Rijsewijk, F.A., Babiuk, L., Pastoret, P.P. (1999) Bovine herpesvirus 1-induced apoptotic cell death: role of glycoprotein D. *Virology* **257**, 191-7.
47. Zhu, L., Ding, X., Tao, J., Wang, J., Zhao, X., Zhu, G. (2010) Critical role of cholesterol in bovine herpesvirus type 1 infection of MDBK cells. *Vet Microbiol* **144**, 51-7.
48. Bender, F.C., Whitbeck, J.C., Ponce de Leon, M., Lou, H., Eisenberg, R.J., Cohen, G.H. (2003) Specific association of glycoprotein B with lipid rafts during herpes simplex virus entry. *J Virol* **77**, 9542-52.
49. Hambleton, S., Steinberg, S.P., Gershon, M.D., Gershon, A.A. (2007) Cholesterol dependence of varicella-zoster virion entry into target cells. *J Virol* **81**, 7548-58.
50. Desplanques, A.S., Nauwynck, H.J., Vercauteren, D., Geens, T., Favoreel, H.W. (2008) Plasma membrane cholesterol is required for efficient pseudorabies virus entry. *Virology* **376**, 339-45.
51. Fraefel, C., Wirth, U.V., Vogt, B., Schwyzer, M. (1993) Immediate-early transcription over covalently joined genome ends of bovine herpesvirus 1: the circ gene. *J Virol* **67**, 1328-33.
52. Fuchs, W., Klupp, B.G., Granzow, H., Osterrieder, N., Mettenleiter, T.C. (2002) The interacting UL31 and UL34 gene products of pseudorabies virus are involved in egress from the host-cell nucleus and represent components of primary enveloped but not mature virions. *J Virol* **76**, 364-78.
53. Mettenleiter, T.C. (2004) Budding events in herpesvirus morphogenesis. *Virus Res* **106**, 167-80.

54. Loret, S., Guay, G., Lippe, R. (2008) Comprehensive characterization of extracellular herpes simplex virus type 1 virions. *J Virol* **82**, 8605-18.
55. Gibbs, E.P.J., Rweyemamu, M.M. (1977) Bovine Herpesviruses. Part 1: Bovine herpesvirus 1. *Veterinary Bulletin* **47**, 317-343.
56. Tikoo, S.K., Campos, M., Babiuk, L.A. (1995) Bovine herpesvirus 1 (BHV-1): biology, pathogenesis, and control. *Adv Virus Res* **45**, 191-223.
57. Ludwig, H. (1983) Bovine Herpesviruses. In *The herpesviruses*, Volume 2 (B. Roizman, ed) Plenum Press, New York, NY 135-214.
58. Hammerschmidt, W., Ludwig, H., Buhk, H.J. (1988) Specificity of cleavage in replicative-form DNA of bovine herpesvirus 1. *J Virol* **62**, 1355-63.
59. Muylkens, B., Thiry, J., Kirten, P., Schynts, F., Thiry, E. (2007) Bovine herpesvirus 1 infection and infectious bovine rhinotracheitis. *Vet Res* **38**, 181-209.
60. Mayfield, J.E., Good, P.J., VanOort, H.J., Campbell, A.R., Reed, D.E. (1983) Cloning and cleavage site mapping of DNA from bovine herpesvirus 1 (Cooper strain). *J Virol* **47**, 259-64.
61. Schwyzer, M., Ackermann, M. (1996) Molecular virology of ruminant herpesviruses. *Vet Microbiol* **53**, 17-29.
62. Schynts, F., McVoy, M.A., Meurens, F., Detry, B., Epstein, A.L., Thiry, E. (2003) The structures of bovine herpesvirus 1 virion and concatemeric DNA: implications for cleavage and packaging of herpesvirus genomes. *Virology* **314**, 326-35.
63. Jacob, R.J., Morse, L.S., Roizman, B. (1979) Anatomy of herpes simplex virus DNA. XII. Accumulation of head-to-tail concatemers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA. *J Virol* **29**, 448-57.
64. Delhon, G., Moraes, M.P., Lu, Z., Afonso, C.L., Flores, E.F., Weiblen, R., Kutish, G.F., Rock, D.L. (2003) Genome of bovine herpesvirus 5. *J Virol* **77**, 10339-47.
65. Robinson, K.E., Meers, J., Gravel, J.L., McCarthy, F.M., Mahony, T.J. (2008) The essential and non-essential genes of Bovine herpesvirus 1. *J Gen Virol* **89**, 2851-63.
66. Spurzem, J.R., Raz, M., Ito, H., Kelling, C.L., Stine, L.C., Romberger, D.J., Rennard, S.I. (1995) Bovine herpesvirus-1 infection reduces bronchial epithelial cell migration to extracellular matrix proteins. *Am J Physiol* **268**, L214-20.
67. Babiuk, L.A., van Drunen Littel-van den Hurk, S., Tikoo, S.K. (1996) Immunology of bovine herpesvirus 1 infection. *Vet Microbiol* **53**, 31-42.
68. Baluyut, C.S., Simonson, R.R., Bemrick, W.J., Maheswaran, S.K. (1981) Interaction of Pasteurella haemolytica with bovine neutrophils: identification and partial characterization of a cytotoxin. *Am J Vet Res* **42**, 1920-6.
69. Haig, S.-J. (2011) Adherence of Mannheimia haemolytica to ovine bronchial epithelial cells. *Bioscience Horizons* **4**, 50-60.
70. Cusack, P.M., McMeniman, N., Lean, I.J. (2003) The medicine and epidemiology of bovine respiratory disease in feedlots. *Aust Vet J* **81**, 480-7.
71. Yates, W.D. (1982) A review of infectious bovine rhinotracheitis, shipping fever pneumonia and viral-bacterial synergism in respiratory disease of cattle. *Can J Comp Med* **46**, 225-63.
72. Enquist, L.W., Husak, P.J., Banfield, B.W., Smith, G.A. (1998) Infection and spread of alphaherpesviruses in the nervous system. *Adv Virus Res* **51**, 237-347.
73. Srikumaran, S., Kelling, C.L., Ambagala, A. (2007) Immune evasion by pathogens of bovine respiratory disease complex. *Anim Health Res Rev* **8**, 215-29.

74. Fuchs, M., Hubert, P., Detterer, J., Rziha, H.J. (1999) Detection of bovine herpesvirus type 1 in blood from naturally infected cattle by using a sensitive PCR that discriminates between wild-type virus and virus lacking glycoprotein E. *J Clin Microbiol* **37**, 2498-507.
75. Carter, J.J., Weinberg, A.D., Pollard, A., Reeves, R., Magnuson, J.A., Magnuson, N.S. (1989) Inhibition of T-lymphocyte mitogenic responses and effects on cell functions by bovine herpesvirus 1. *J Virol* **63**, 1525-30.
76. Hutchings, D.L., Campos, M., Qualtiere, L., Babiuk, L.A. (1990) Inhibition of antigen-induced and interleukin-2-induced proliferation of bovine peripheral blood leukocytes by inactivated bovine herpes virus 1. *J Virol* **64**, 4146-51.
77. Winkler, M.T., Doster, A., Jones, C. (1999) Bovine herpesvirus 1 can infect CD4(+) T lymphocytes and induce programmed cell death during acute infection of cattle. *J Virol* **73**, 8657-68.
78. Lipinska, A.D., Koppers-Lalic, D., Rychlowski, M., Admiraal, P., Rijsewijk, F.A., Bienkowska-Szewczyk, K., Wiertz, E.J. (2006) Bovine herpesvirus 1 UL49.5 protein inhibits the transporter associated with antigen processing despite complex formation with glycoprotein M. *J Virol* **80**, 5822-32.
79. Deshpande, M.S., Ambagala, T.C., Ambagala, A.P., Kehrli, M.E., Jr., Srikumaran, S. (2002) Bovine CD18 is necessary and sufficient to mediate Mannheimia (Pasteurella) haemolytica leukotoxin-induced cytolysis. *Infect Immun* **70**, 5058-64.
80. Hughes, H.P., Campos, M., McDougall, L., Beskorwayne, T.K., Potter, A.A., Babiuk, L.A. (1994) Regulation of major histocompatibility complex class II expression by Pasteurella haemolytica leukotoxin. *Infect Immun* **62**, 1609-15.
81. Chae, C.H., Gentry, M.J., Confer, A.W., Anderson, G.A. (1990) Resistance to host immune defense mechanisms afforded by capsular material of Pasteurella haemolytica, serotype 1. *Vet Microbiol* **25**, 241-51.
82. Cowley, D.J., Clegg, T.A., Doherty, M.L., More, S.J. (2011) Aspects of bovine herpesvirus-1 infection in dairy and beef herds in the Republic of Ireland. *Acta Vet Scand* **53**, 40.
83. Kahrs, R.F. (1977) Infectious bovine rhinotracheitis: a review and update. *J Am Vet Med Assoc* **171**, 1055-64.
84. Straub, O.C. (1991) BHV1 infections: relevance and spread in Europe. *Comp Immunol Microbiol Infect Dis* **14**, 175-86.
85. Henderson, G., Perng, G.C., Nesburn, A.B., Wechsler, S.L., Jones, C. (2004) The latency-related gene encoded by bovine herpesvirus 1 can suppress caspase 3 and caspase 9 cleavage during productive infection. *J Neurovirol* **10**, 64-70.
86. Inman, M., Zhou, J., Webb, H., Jones, C. (2004) Identification of a novel bovine herpesvirus 1 transcript containing a small open reading frame that is expressed in trigeminal ganglia of latently infected cattle. *J Virol* **78**, 5438-47.
87. Jones, C. (2003) Herpes simplex virus type 1 and bovine herpesvirus 1 latency. *Clin Microbiol Rev* **16**, 79-95.
88. Jones, C., Geiser, V., Henderson, G., Jiang, Y., Meyer, F., Perez, S., Zhang, Y. (2006) Functional analysis of bovine herpesvirus 1 (BHV-1) genes expressed during latency. *Vet Microbiol* **113**, 199-210.
89. Ribble, C.S., Meek, A.H., Jim, G.K., Guichon, P.T. (1995) The pattern of fatal fibrinous pneumonia (shipping fever) affecting calves in a large feedlot in Alberta (1985-1988). *Can Vet J* **36**, 753-7.

90. Castrucci, G., Frigeri, F., Salvatori, D., Ferrari, M., Sardonini, Q., Cassai, E., Lo, D.M., Rotola, A., Angelini, R. (2002) Vaccination of calves against bovine herpesvirus-1: assessment of the protective value of eight vaccines. *Comp Immunol Microbiol Infect Dis* **25**, 29-41.
91. Gagea, M.I., Bateman, K.G., van Dreumel, T., McEwen, B.J., Carman, S., Archambault, M., Shanahan, R.A., Caswell, J.L. (2006) Diseases and pathogens associated with mortality in Ontario beef feedlots. *J Vet Diagn Invest* **18**, 18-28.
92. Loneragan, G.H., Dargatz, D.A., Morley, P.S., Smith, M.A. (2001) Trends in mortality ratios among cattle in US feedlots. *J Am Vet Med Assoc* **219**, 1122-7.
93. Martin, S.W., Meek, A.H., Davis, D.G., Johnson, J.A., Curtis, R.A. (1982) Factors associated with mortality and treatment costs in feedlot calves: the Bruce County Beef Project, years 1978, 1979, 1980. *Can J Comp Med* **46**, 341-9.
94. Strube, W., Auer, S., Block, W., Heinen, E., Kretzdorn, D., Rodenbach, C., Schmeer, N. (1996) A gE deleted infectious bovine rhinotracheitis marker vaccine for use in improved bovine herpesvirus 1 control programs. *Vet Microbiol* **53**, 181-9.
95. Yates, W.D., Babiuk, L.A., Jericho, K.W. (1983) Viral-bacterial pneumonia in calves: duration of the interaction between bovine herpesvirus 1 and Pasteurella haemolytica. *Can J Comp Med* **47**, 257-64.
96. Ackermann, M., Engels, M. (2006) Pro and contra IBR-eradication. *Vet Microbiol* **113**, 293-302.
97. Thiry, E., Muylkens, B., Meurens, F., Gogev, S., Thiry, J., Vanderplasschen, A., Schynts, F. (2006) Recombination in the alphaherpesvirus bovine herpesvirus 1. *Vet Microbiol* **113**, 171-7.
98. van Drunen Littel-van den Hurk, S., Tikoo, S.K., van den Hurk, J.V., Babiuk, L.A., Van Donkersgoed, J. (1997) Protective immunity in cattle following vaccination with conventional and marker bovine herpesvirus-1 (BHV1) vaccines. *Vaccine* **15**, 36-44.
99. van Drunen Littel-van den Hurk, S., Myers, D., Doig, P.A., Karvonen, B., Habermehl, M., Babiuk, L.A., Jelinski, M., Van Donkersgoed, J., Schlesinger, K., Rinehart, C. (2001) Identification of a mutant bovine herpesvirus-1 (BHV-1) in post-arrival outbreaks of IBR in feedlot calves and protection with conventional vaccination. *Can J Vet Res* **65**, 81-8.
100. Ellis, J., Waldner, C., Rhodes, C., Ricketts, V. (2005) Longevity of protective immunity to experimental bovine herpesvirus-1 infection following inoculation with a combination modified-live virus vaccine in beef calves. *J Am Vet Med Assoc* **227**, 123-8.
101. van Drunen Littel-van den Hurk, S., Tikoo, S.K., Liang, X., Babiuk, L.A. (1993) Bovine herpesvirus-1 vaccines. *Immunol Cell Biol* **71 (Pt 5)**, 405-20.
102. Muylkens, B., Meurens, F., Schynts, F., Farnir, F., Pourchet, A., Bardiau, M., Gogev, S., Thiry, J., Cuisenaire, A., Vanderplasschen, A., Thiry, E. (2006) Intraspecific bovine herpesvirus 1 recombinants carrying glycoprotein E deletion as a vaccine marker are virulent in cattle. *J Gen Virol* **87**, 2149-54.
103. Kalthoff, D., Konig, P., Trapp, S., Beer, M. (2010) Immunization and challenge experiments with a new modified live bovine herpesvirus type 1 marker vaccine prototype adjuvanted with a co-polymer. *Vaccine* **28**, 5871-7.
104. Steinman, R.M. (1991) The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* **9**, 271-96.
105. Hart, D.N. (1997) Dendritic cells: unique leukocyte populations which control the primary immune response. *Blood* **90**, 3245-87.

106. Banchereau, J., Steinman, R.M. (1998) Dendritic cells and the control of immunity. *Nature* **392**, 245-52.
107. Zitvogel, L., Mayordomo, J.I., Tjandrawan, T., DeLeo, A.B., Clarke, M.R., Lotze, M.T., Storkus, W.J. (1996) Therapy of murine tumors with tumor peptide-pulsed dendritic cells: dependence on T cells, B7 costimulation, and T helper cell 1-associated cytokines. *J Exp Med* **183**, 87-97.
108. Tuting, T., Storkus, W.J., Falo, L.D., Jr. (1998) DNA immunization targeting the skin: molecular control of adaptive immunity. *J Invest Dermatol* **111**, 183-8.
109. Rodriguez, A., Regnault, A., Kleijmeer, M., Ricciardi-Castagnoli, P., Amigorena, S. (1999) Selective transport of internalized antigens to the cytosol for MHC class I presentation in dendritic cells. *Nat Cell Biol* **1**, 362-8.
110. Gil-Torregrosa, B.C., Lennon-Dumenil, A.M., Kessler, B., Guermonprez, P., Ploegh, H.L., Fruci, D., van Endert, P., Amigorena, S. (2004) Control of cross-presentation during dendritic cell maturation. *Eur J Immunol* **34**, 398-407.
111. Albert, M.L., Sauter, B., Bhardwaj, N. (1998) Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* **392**, 86-9.
112. Kamath, A.T., Henri, S., Battye, F., Tough, D.F., Shortman, K. (2002) Developmental kinetics and lifespan of dendritic cells in mouse lymphoid organs. *Blood* **100**, 1734-41.
113. Liu, Y.J. (2001) Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. *Cell* **106**, 259-62.
114. Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y.J., Pulendran, B., Palucka, K. (2000) Immunobiology of dendritic cells. *Annu Rev Immunol* **18**, 767-811.
115. Lotze, M.T., Thomson, A.W. (2001) Dendritic Cells: Biology and Clinical Applications. Academic Press 794.
116. Liu, K., Nussenzweig, M.C. (2010) Development and homeostasis of dendritic cells. *Eur J Immunol* **40**, 2099-102.
117. Van de Walle, G.R., Cox, E., Nauwynck, H., Favoreel, H.W. (2009) The role of dendritic cells in alphaherpesvirus infections: archetypes and paradigms. *Rev Med Virol* **19**, 338-58.
118. Auffray, C., Emre, Y., Geissmann, F. (2008) Homeostasis of dendritic cell pool in lymphoid organs. *Nat Immunol* **9**, 584-6.
119. Shortman, K., Liu, Y.J. (2002) Mouse and human dendritic cell subtypes. *Nat Rev Immunol* **2**, 151-61.
120. van de Ven, R., van den Hout, M.F., Lindenberg, J.J., Sluijter, B.J., van Leeuwen, P.A., Lougheed, S.M., Meijer, S., van den Tol, M.P., Scheper, R.J., de Gruijl, T.D. (2011) Characterization of four conventional dendritic cell subsets in human skin-draining lymph nodes in relation to T-cell activation. *Blood* **118**, 2502-10.
121. Shortman, K., Naik, S.H. (2007) Steady-state and inflammatory dendritic-cell development. *Nat Rev Immunol* **7**, 19-30.
122. Dalod, M., Chelbi, R., Malissen, B., Lawrence, T. (2014) Dendritic cell maturation: functional specialization through signaling specificity and transcriptional programming. *EMBO J* **33**, 1104-1116.
123. Richters, C.D., Mayen, I., Havenith, C.E., Beelen, R.H., Kamperdijk, E.W. (2002) Rat monocyte-derived dendritic cells function and migrate in the same way as isolated tissue dendritic cells. *J Leukoc Biol* **71**, 582-7.

124. Fitzgerald-Bocarsly, P., Dai, J., Singh, S. (2008) Plasmacytoid dendritic cells and type I IFN: 50 years of convergent history. *Cytokine Growth Factor Rev* **19**, 3-19.
125. Garcia-Sastre, A., Biron, C.A. (2006) Type 1 interferons and the virus-host relationship: a lesson in detente. *Science* **312**, 879-82.
126. Schmidt, S.V., Nino-Castro, A.C., Schultze, J.L. (2012) Regulatory dendritic cells: there is more than just immune activation. *Front Immunol* **3**, 274.
127. Steinman, R.M., Nussenzweig, M.C. (2002) Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance. *Proc Natl Acad Sci U S A* **99**, 351-8.
128. Cyster, J.G. (1999) Chemokines and the homing of dendritic cells to the T cell areas of lymphoid organs. *J Exp Med* **189**, 447-50.
129. Gouwy, M., Struyf, S., Proost, P., Van Damme, J. (2005) Synergy in cytokine and chemokine networks amplifies the inflammatory response. *Cytokine Growth Factor Rev* **16**, 561-80.
130. Svensson, M., Pfeifer, J., Stockinger, B., Wick, M.J. (1997) Bacterial antigen delivery systems: phagocytic processing of bacterial antigens for MHC-I and MHC-II presentation to T cells. *Behring Inst Mitt*, 197-211.
131. Reis e Sousa, C., Stahl, P.D., Austyn, J.M. (1993) Phagocytosis of antigens by Langerhans cells in vitro. *J Exp Med* **178**, 509-19.
132. Sallusto, F., Cella, M., Danieli, C., Lanzavecchia, A. (1995) Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J Exp Med* **182**, 389-400.
133. Kato, M., Neil, T.K., Clark, G.J., Morris, C.M., Sorg, R.V., Hart, D.N. (1998) cDNA cloning of human DEC-205, a putative antigen-uptake receptor on dendritic cells. *Immunogenetics* **47**, 442-50.
134. Jiang, W., Swiggard, W.J., Heufler, C., Peng, M., Mirza, A., Steinman, R.M., Nussenzweig, M.C. (1995) The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. *Nature* **375**, 151-5.
135. Steinman, R.M., Pope, M. (2002) Exploiting dendritic cells to improve vaccine efficacy. *J Clin Invest* **109**, 1519-26.
136. Pardoll, D.M. (2002) Spinning molecular immunology into successful immunotherapy. *Nat Rev Immunol* **2**, 227-38.
137. Luster, A.D. (2002) The role of chemokines in linking innate and adaptive immunity. *Curr Opin Immunol* **14**, 129-35.
138. De Haes, W., Pollard, C., Vanham, G., Rejman, J. (2012) "Wrapped Up" Vaccines in the Context of HIV-1 Immunotherapy. In *Immunodeficiency* (K. Metodiev, ed) InTech.
139. Kitajima, T., Caceres-Dittmar, G., Tapia, F.J., Jester, J., Bergstresser, P.R., Takashima, A. (1996) T cell-mediated terminal maturation of dendritic cells: loss of adhesive and phagocytotic capacities. *J Immunol* **157**, 2340-7.
140. Wimmers, F., Schreibelt, G., Skold, A.E., Figdor, C.G., De Vries, I.J. (2014) Paradigm Shift in Dendritic Cell-Based Immunotherapy: From Generated Monocyte-Derived DCs to Naturally Circulating DC Subsets. *Front Immunol* **5**, 165.
141. Summers deLuca, L., Gommerman, J.L. (2012) Fine-tuning of dendritic cell biology by the TNF superfamily. *Nat Rev Immunol* **12**, 339-51.
142. Kalinski, P., Smits, H.H., Schuitemaker, J.H., Vieira, P.L., van Eijk, M., de Jong, E.C., Wierenga, E.A., Kapsenberg, M.L. (2000) IL-4 is a mediator of IL-12p70 induction by

human Th2 cells: reversal of polarized Th2 phenotype by dendritic cells. *J Immunol* **165**, 1877-81.
143. Zietara, N., Lyszkiewicz, M., Puchalka, J., Pei, G., Gutierrez, M.G., Lienenklaus, S., Hobeika, E., Reth, M., Martins dos Santos, V.A., Krueger, A., Weiss, S. (2013) Immunoglobulins drive terminal maturation of splenic dendritic cells. *Proc Natl Acad Sci U S A* **110**, 2282-7.
144. Kato, K., Takaue, Y., Wakasugi, H. (2001) T-cell-conditioned medium efficiently induces the maturation and function of human dendritic cells. *J Leukoc Biol* **70**, 941-9.
145. Roitt, I., Brostoff, J., Male, D. (2001) *Immunology*. Mosby, Edinburgh.
146. Kobelt, D., Lechmann, M., Steinkasserer, A. (2003) The interaction between dendritic cells and herpes simplex virus-1. *Curr Top Microbiol Immunol* **276**, 145-61.
147. de Jong, M.A., de Witte, L., Bolmstedt, A., van Kooyk, Y., Geijtenbeek, T.B. (2008) Dendritic cells mediate herpes simplex virus infection and transmission through the C-type lectin DC-SIGN. *J Gen Virol* **89**, 2398-409.
148. Kummer, M., Turza, N.M., Muhl-Zurbes, P., Lechmann, M., Boutell, C., Coffin, R.S., Everett, R.D., Steinkasserer, A., Prechtel, A.T. (2007) Herpes simplex virus type 1 induces CD83 degradation in mature dendritic cells with immediate-early kinetics via the cellular proteasome. *J Virol* **81**, 6326-38.
149. Esser, M.T., Marchese, R.D., Kierstead, L.S., Tussey, L.G., Wang, F., Chirmule, N., Washabaugh, M.W. (2003) Memory T cells and vaccines. *Vaccine* **21**, 419-30.
150. Seya, T. (2008) Toll-like receptor and pattern sensing for evoking immune response. *Adv Drug Deliv Rev* **60**, 779-81.
151. Asselin-Paturel, C., Boonstra, A., Dalod, M., Durand, I., Yessaad, N., Dezutter-Dambuyant, C., Vicari, A., O'Garra, A., Biron, C., Briere, F., Trinchieri, G. (2001) Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. *Nat Immunol* **2**, 1144-50.
152. Pirhonen, J., Sareneva, T., Kurimoto, M., Julkunen, I., Matikainen, S. (1999) Virus infection activates IL-1 beta and IL-18 production in human macrophages by a caspase-1-dependent pathway. *J Immunol* **162**, 7322-9.
153. Bjorck, P. (2004) Dendritic cells exposed to herpes simplex virus in vivo do not produce IFN-alpha after rechallenge with virus in vitro and exhibit decreased T cell alloreactivity. *J Immunol* **172**, 5396-404.
154. Eisemann, J., Muhl-Zurbes, P., Steinkasserer, A., Kummer, M. (2007) Infection of mature dendritic cells with herpes simplex virus type 1 interferes with the interferon signaling pathway. *Immunobiology* **212**, 877-86.
155. Prechtel, A.T., Turza, N.M., Kobelt, D.J., Eisemann, J.I., Coffin, R.S., McGrath, Y., Hacker, C., Ju, X., Zenke, M., Steinkasserer, A. (2005) Infection of mature dendritic cells with herpes simplex virus type 1 dramatically reduces lymphoid chemokine-mediated migration. *J Gen Virol* **86**, 1645-57.
156. Mandelboim, O., Lieberman, N., Lev, M., Paul, L., Arnon, T.I., Bushkin, Y., Davis, D.M., Strominger, J.L., Yewdell, J.W., Porgador, A. (2001) Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature* **409**, 1055-60.
157. Pollara, G., Speidel, K., Samady, L., Rajpopat, M., McGrath, Y., Ledermann, J., Coffin, R.S., Katz, D.R., Chain, B. (2003) Herpes simplex virus infection of dendritic cells: balance among activation, inhibition, and immunity. *J Infect Dis* **187**, 165-78.

158. Nopora, K., Bernhard, C.A., Ried, C., Castello, A.A., Murphy, K.M., Marconi, P., Koszinowski, U., Brocker, T. (2012) MHC class I cross-presentation by dendritic cells counteracts viral immune evasion. *Front Immunol* **3**, 348.
159. Piqueras, B., Connolly, J., Freitas, H., Palucka, A.K., Banchereau, J. (2006) Upon viral exposure, myeloid and plasmacytoid dendritic cells produce 3 waves of distinct chemokines to recruit immune effectors. *Blood* **107**, 2613-8.
160. Schwartz, R.H. (2003) T cell anergy. *Annu Rev Immunol* **21**, 305-34.
161. Schiavo, R., Baatar, D., Olkhanud, P., Indig, F.E., Restifo, N., Taub, D., Biragyn, A. (2006) Chemokine receptor targeting efficiently directs antigens to MHC class I pathways and elicits antigen-specific CD8+ T-cell responses. *Blood* **107**, 4597-605.
162. Dubois, B., Massacrier, C., Vanbervliet, B., Fayette, J., Briere, F., Banchereau, J., Caux, C. (1998) Critical role of IL-12 in dendritic cell-induced differentiation of naive B lymphocytes. *J Immunol* **161**, 2223-31.
163. Dubois, B., Vanbervliet, B., Fayette, J., Massacrier, C., Van Kooten, C., Briere, F., Banchereau, J., Caux, C. (1997) Dendritic cells enhance growth and differentiation of CD40-activated B lymphocytes. *J Exp Med* **185**, 941-51.
164. Moore, P.A., Belvedere, O., Orr, A., Pieri, K., LaFleur, D.W., Feng, P., Soppet, D., Charters, M., Gentz, R., Parmelee, D., Li, Y., Galperina, O., Giri, J., Roschke, V., Nardelli, B., Carrell, J., Sosnovtseva, S., Greenfield, W., Ruben, S.M., Olsen, H.S., Fikes, J., Hilbert, D.M. (1999) BLyS: member of the tumor necrosis factor family and B lymphocyte stimulator. *Science* **285**, 260-3.
165. Schneider, P., MacKay, F., Steiner, V., Hofmann, K., Bodmer, J.L., Holler, N., Ambrose, C., Lawton, P., Bixler, S., Acha-Orbea, H., Valmori, D., Romero, P., Werner-Favre, C., Zubler, R.H., Browning, J.L., Tschopp, J. (1999) BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth. *J Exp Med* **189**, 1747-56.
166. Thomma, B.P., Cammue, B.P., Thevissen, K. (2002) Plant defensins. *Planta* **216**, 193-202.
167. Lay, F.T., Anderson, M.A. (2005) Defensins--components of the innate immune system in plants. *Curr Protein Pept Sci* **6**, 85-101.
168. Hoffmann, J.A., Hetru, C. (1992) Insect defensins: inducible antibacterial peptides. *Immunol Today* **13**, 411-5.
169. Froy, O., Gurevitz, M. (2003) Arthropod and mollusk defensins--evolution by exon-shuffling. *Trends Genet* **19**, 684-7.
170. Bulet, P., Stocklin, R., Menin, L. (2004) Anti-microbial peptides: from invertebrates to vertebrates. *Immunol Rev* **198**, 169-84.
171. Boman, H.G. (1998) Gene-encoded peptide antibiotics and the concept of innate immunity: an update review. *Scand J Immunol* **48**, 15-25.
172. Hilchie, A.L., Wuerth, K., Hancock, R.E. (2013) Immune modulation by multifaceted cationic host defense (antimicrobial) peptides. *Nat Chem Biol* **9**, 761-8.
173. Mookherjee, N., Hancock, R.E. (2007) Cationic host defence peptides: innate immune regulatory peptides as a novel approach for treating infections. *Cell Mol Life Sci* **64**, 922-33.
174. Mulder, K.C., Lima, L.A., Miranda, V.J., Dias, S.C., Franco, O.L. (2013) Current scenario of peptide-based drugs: the key roles of cationic antitumor and antiviral peptides. *Front Microbiol* **4**, 321.

175. Selsted, M.E., Ouellette, A.J. (2005) Mammalian defensins in the antimicrobial immune response. *Nat Immunol* **6**, 551-7.
176. Lehrer, R.I. (2004) Primate defensins. *Nat Rev Microbiol* **2**, 727-38.
177. Lehrer, R.I., Ganz, T. (2002) Cathelicidins: a family of endogenous antimicrobial peptides. *Curr Opin Hematol* **9**, 18-22.
178. Durr, U.H., Sudheendra, U.S., Ramamoorthy, A. (2006) LL-37, the only human member of the cathelicidin family of antimicrobial peptides. *Biochim Biophys Acta* **1758**, 1408-25.
179. Nizet, V., Gallo, R.L. (2003) Cathelicidins and innate defense against invasive bacterial infection. *Scand J Infect Dis* **35**, 670-6.
180. Zanetti, M. (2004) Cathelicidins, multifunctional peptides of the innate immunity. *J Leukoc Biol* **75**, 39-48.
181. Tran, D., Tran, P.A., Tang, Y.Q., Yuan, J., Cole, T., Selsted, M.E. (2002) Homodimeric theta-defensins from rhesus macaque leukocytes: isolation, synthesis, antimicrobial activities, and bacterial binding properties of the cyclic peptides. *J Biol Chem* **277**, 3079-84.
182. Kaiser, V., Diamond, G. (2000) Expression of mammalian defensin genes. *J Leukoc Biol* **68**, 779-84.
183. Yang, D., Biragyn, A., Hoover, D.M., Lubkowski, J., Oppenheim, J.J. (2004) Multiple roles of antimicrobial defensins, cathelicidins, and eosinophil-derived neurotoxin in host defense. *Annu Rev Immunol* **22**, 181-215.
184. Ganz, T. (2003) Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol* **3**, 710-20.
185. Diamond, G., Bevins, C.L. (1998) beta-Defensins: endogenous antibiotics of the innate host defense response. *Clin Immunol Immunopathol* **88**, 221-5.
186. Patterson-Delafield, J., Martinez, R.J., Lehrer, R.I. (1980) Microbicidal cationic proteins in rabbit alveolar macrophages: a potential host defense mechanism. *Infect Immun* **30**, 180-92.
187. Lehrer, R.I., Daher, K., Ganz, T., Selsted, M.E. (1985) Direct inactivation of viruses by MCP-1 and MCP-2, natural peptide antibiotics from rabbit leukocytes. *J Virol* **54**, 467-72.
188. Lehrer, R.I., Lu, W. (2012) alpha-Defensins in human innate immunity. *Immunol Rev* **245**, 84-112.
189. Selsted, M.E., Brown, D.M., DeLange, R.J., Harwig, S.S., Lehrer, R.I. (1985) Primary structures of six antimicrobial peptides of rabbit peritoneal neutrophils. *J Biol Chem* **260**, 4579-84.
190. Ganz, T., Selsted, M.E., Szklarek, D., Harwig, S.S., Daher, K., Bainton, D.F., Lehrer, R.I. (1985) Defensins. Natural peptide antibiotics of human neutrophils. *J Clin Invest* **76**, 1427-35.
191. Cunliffe, R.N. (2003) Alpha-defensins in the gastrointestinal tract. *Mol Immunol* **40**, 463-7.
192. Quayle, A.J., Porter, E.M., Nussbaum, A.A., Wang, Y.M., Brabec, C., Yip, K.P., Mok, S.C. (1998) Gene expression, immunolocalization, and secretion of human defensin-5 in human female reproductive tract. *Am J Pathol* **152**, 1247-58.
193. Eisenhauer, P.B., Harwig, S.S., Lehrer, R.I. (1992) Cryptdins: antimicrobial defensins of the murine small intestine. *Infect Immun* **60**, 3556-65.

194. Ouellette, A.J., Hsieh, M.M., Nosek, M.T., Cano-Gauci, D.F., Huttner, K.M., Buick, R.N., Selsted, M.E. (1994) Mouse Paneth cell defensins: primary structures and antibacterial activities of numerous cryptdin isoforms. *Infect Immun* **62**, 5040-7.
195. Eisenhauer, P.B., Lehrer, R.I. (1992) Mouse neutrophils lack defensins. *Infect Immun* **60**, 3446-7.
196. Ouellette, A.J., Miller, S.I., Henschen, A.H., Selsted, M.E. (1992) Purification and primary structure of murine cryptdin-1, a Paneth cell defensin. *FEBS Lett* **304**, 146-8.
197. Ouellette, A.J. (1997) Paneth cells and innate immunity in the crypt microenvironment. *Gastroenterology* **113**, 1779-84.
198. Fjell, C.D., Jenssen, H., Fries, P., Aich, P., Griebel, P., Hilpert, K., Hancock, R.E., Cherkasov, A. (2008) Identification of novel host defense peptides and the absence of alpha-defensins in the bovine genome. *Proteins* **73**, 420-30.
199. Diamond, G., Zasloff, M., Eck, H., Brasseur, M., Maloy, W.L., Bevins, C.L. (1991) Tracheal antimicrobial peptide, a cysteine-rich peptide from mammalian tracheal mucosa: peptide isolation and cloning of a cDNA. *Proc Natl Acad Sci U S A* **88**, 3952-6.
200. Schonwetter, B.S., Stolzenberg, E.D., Zasloff, M.A. (1995) Epithelial antibiotics induced at sites of inflammation. *Science* **267**, 1645-8.
201. Tarver, A.P., Clark, D.P., Diamond, G., Russell, J.P., Erdjument-Bromage, H., Tempst, P., Cohen, K.S., Jones, D.E., Sweeney, R.W., Wines, M., Hwang, S., Bevins, C.L. (1998) Enteric beta-defensin: molecular cloning and characterization of a gene with inducible intestinal epithelial cell expression associated with Cryptosporidium parvum infection. *Infect Immun* **66**, 1045-56.
202. Michaelson, D., Rayner, J., Couto, M., Ganz, T. (1992) Cationic defensins arise from charge-neutralized propeptides: a mechanism for avoiding leukocyte autocytotoxicity? *J Leukoc Biol* **51**, 634-9.
203. Schutte, B.C., McCray, P.B., Jr. (2002) [beta]-defensins in lung host defense. *Annu Rev Physiol* **64**, 709-48.
204. Patil, A., Hughes, A.L., Zhang, G. (2004) Rapid evolution and diversification of mammalian alpha-defensins as revealed by comparative analysis of rodent and primate genes. *Physiol Genomics* **20**, 1-11.
205. Lynn, D.J., Lloyd, A.T., Fares, M.A., O'Farrelly, C. (2004) Evidence of positively selected sites in mammalian alpha-defensins. *Mol Biol Evol* **21**, 819-27.
206. Jia, H.P., Schutte, B.C., Schudy, A., Linzmeier, R., Guthmiller, J.M., Johnson, G.K., Tack, B.F., Mitros, J.P., Rosenthal, A., Ganz, T., McCray, P.B., Jr. (2001) Discovery of new human beta-defensins using a genomics-based approach. *Gene* **263**, 211-8.
207. Ali, R.S., Falconer, A., Ikram, M., Bissett, C.E., Cerio, R., Quinn, A.G. (2001) Expression of the peptide antibiotics human beta defensin-1 and human beta defensin-2 in normal human skin. *J Invest Dermatol* **117**, 106-11.
208. Pazgier, M., Hoover, D.M., Yang, D., Lu, W., Lubkowski, J. (2006) Human beta-defensins. *Cell Mol Life Sci* **63**, 1294-313.
209. Huang, L., Ching, C.B., Jiang, R., Leong, S.S. (2008) Production of bioactive human beta-defensin 5 and 6 in Escherichia coli by soluble fusion expression. *Protein Expr Purif* **61**, 168-74.
210. Yamaguchi, Y., Nagase, T., Makita, R., Fukuhara, S., Tomita, T., Tominaga, T., Kurihara, H., Ouchi, Y. (2002) Identification of multiple novel epididymis-specific beta-defensin isoforms in humans and mice. *J Immunol* **169**, 2516-23.

211. Sun, L., Finnegan, C.M., Kish-Catalone, T., Blumenthal, R., Garzino-Demo, P., La Terra Maggiore, G.M., Berrone, S., Kleinman, C., Wu, Z., Abdelwahab, S., Lu, W., Garzino-Demo, A. (2005) Human beta-defensins suppress human immunodeficiency virus infection: potential role in mucosal protection. *J Virol* **79**, 14318-29.
212. Quinones-Mateu, M.E., Lederman, M.M., Feng, Z., Chakraborty, B., Weber, J., Rangel, H.R., Marotta, M.L., Mirza, M., Jiang, B., Kiser, P., Medvik, K., Sieg, S.F., Weinberg, A. (2003) Human epithelial beta-defensins 2 and 3 inhibit HIV-1 replication. *Aids* **17**, F39-48.
213. Ferris, L.K., Mburu, Y.K., Mathers, A.R., Fluharty, E.R., Larregina, A.T., Ferris, R.L., Falo, L.D., Jr. (2013) Human beta-defensin 3 induces maturation of human langerhans cell-like dendritic cells: an antimicrobial peptide that functions as an endogenous adjuvant. *J Invest Dermatol* **133**, 460-8.
214. Yang, D., Chertov, O., Bykovskaia, S.N., Chen, Q., Buffo, M.J., Shogan, J., Anderson, M., Schroder, J.M., Wang, J.M., Howard, O.M., Oppenheim, J.J. (1999) Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. *Science* **286**, 525-8.
215. Rohrl, J., Yang, D., Oppenheim, J.J., Hehlgans, T. (2010) Human beta-defensin 2 and 3 and their mouse orthologs induce chemotaxis through interaction with CCR2. *J Immunol* **184**, 6688-94.
216. Biragyn, A., Ruffini, P.A., Leifer, C.A., Klyushnenkova, E., Shakhov, A., Chertov, O., Shirakawa, A.K., Farber, J.M., Segal, D.M., Oppenheim, J.J., Kwak, L.W. (2002) Toll-like receptor 4-dependent activation of dendritic cells by beta-defensin 2. *Science* **298**, 1025-9.
217. Biragyn, A., Surenhu, M., Yang, D., Ruffini, P.A., Haines, B.A., Klyushnenkova, E., Oppenheim, J.J., Kwak, L.W. (2001) Mediators of innate immunity that target immature, but not mature, dendritic cells induce antitumor immunity when genetically fused with nonimmunogenic tumor antigens. *J Immunol* **167**, 6644-53.
218. Biragyn, A., Belyakov, I.M., Chow, Y.H., Dimitrov, D.S., Berzofsky, J.A., Kwak, L.W. (2002) DNA vaccines encoding human immunodeficiency virus-1 glycoprotein 120 fusions with proinflammatory chemoattractants induce systemic and mucosal immune responses. *Blood* **100**, 1153-9.
219. Selsted, M.E., Tang, Y.Q., Morris, W.L., McGuire, P.A., Novotny, M.J., Smith, W., Henschen, A.H., Cullor, J.S. (1993) Purification, primary structures, and antibacterial activities of beta-defensins, a new family of antimicrobial peptides from bovine neutrophils. *J Biol Chem* **268**, 6641-8.
220. Mallow, E.B., Harris, A., Salzman, N., Russell, J.P., DeBerardinis, R.J., Ruchelli, E., Bevins, C.L. (1996) Human enteric defensins. Gene structure and developmental expression. *J Biol Chem* **271**, 4038-45.
221. Huttner, K.M., Lambeth, M.R., Burkin, H.R., Burkin, D.J., Broad, T.E. (1998) Localization and genomic organization of sheep antimicrobial peptide genes. *Gene* **206**, 85-91.
222. Diamond, G., Russell, J.P., Bevins, C.L. (1996) Inducible expression of an antibiotic peptide gene in lipopolysaccharide-challenged tracheal epithelial cells. *Proc Natl Acad Sci U S A* **93**, 5156-60.
223. Diamond, G., Bevins, C.L. (1994) Endotoxin upregulates expression of an antimicrobial peptide gene in mammalian airway epithelial cells. *Chest* **105**, 51S-52S.

224. Russell, J.P., Diamond, G., Tarver, A.P., Scanlin, T.F., Bevins, C.L. (1996) Coordinate induction of two antibiotic genes in tracheal epithelial cells exposed to the inflammatory mediators lipopolysaccharide and tumor necrosis factor alpha. *Infect Immun* **64**, 1565-8.
225. Ganz, T., Lehrer, R.I. (1994) Defensins. *Curr Opin Immunol* **6**, 584-9.
226. Stolzenberg, E.D., Anderson, G.M., Ackermann, M.R., Whitlock, R.H., Zasloff, M. (1997) Epithelial antibiotic induced in states of disease. *Proc Natl Acad Sci U S A* **94**, 8686-90.
227. Luenser, K., Ludwig, A. (2005) Variability and evolution of bovine beta-defensin genes. *Genes Immun* **6**, 115-22.
228. Aono, S., Li, C., Zhang, G., Kemppainen, R.J., Gard, J., Lu, W., Hu, X., Schwartz, D.D., Morrison, E.E., Dykstra, C., Shi, J. (2006) Molecular and functional characterization of bovine beta-defensin-1. *Vet Immunol Immunopathol* **113**, 181-90.
229. Cole, A.M., Ganz, T., Liese, A.M., Burdick, M.D., Liu, L., Strieter, R.M. (2001) Cutting edge: IFN-inducible ELR- CXC chemokines display defensin-like antimicrobial activity. *J Immunol* **167**, 623-7.
230. Niyonsaba, F., Someya, A., Hirata, M., Ogawa, H., Nagaoka, I. (2001) Evaluation of the effects of peptide antibiotics human beta-defensins-1/-2 and LL-37 on histamine release and prostaglandin D(2) production from mast cells. *Eur J Immunol* **31**, 1066-75.
231. Niyonsaba, F., Iwabuchi, K., Matsuda, H., Ogawa, H., Nagaoka, I. (2002) Epithelial cell-derived human beta-defensin-2 acts as a chemotaxin for mast cells through a pertussis toxin-sensitive and phospholipase C-dependent pathway. *Int Immunol* **14**, 421-6.
232. Niyonsaba, F., Ogawa, H., Nagaoka, I. (2004) Human beta-defensin-2 functions as a chemotactic agent for tumour necrosis factor-alpha-treated human neutrophils. *Immunology* **111**, 273-81.
233. Chen, X., Niyonsaba, F., Ushio, H., Hara, M., Yokoi, H., Matsumoto, K., Saito, H., Nagaoka, I., Ikeda, S., Okumura, K., Ogawa, H. (2007) Antimicrobial peptides human beta-defensin (hBD)-3 and hBD-4 activate mast cells and increase skin vascular permeability. *Eur J Immunol* **37**, 434-44.
234. Seo, E.S., Blaum, B.S., Vargues, T., De Cecco, M., Deakin, J.A., Lyon, M., Barran, P.E., Campopiano, D.J., Uhrin, D. (2010) Interaction of human beta-defensin 2 (HBD2) with glycosaminoglycans. *Biochemistry* **49**, 10486-95.
235. Lillard, J.W., Jr., Boyaka, P.N., Chertov, O., Oppenheim, J.J., McGhee, J.R. (1999) Mechanisms for induction of acquired host immunity by neutrophil peptide defensins. *Proc Natl Acad Sci U S A* **96**, 651-6.
236. Niyonsaba, F., Ushio, H., Nakano, N., Ng, W., Sayama, K., Hashimoto, K., Nagaoka, I., Okumura, K., Ogawa, H. (2007) Antimicrobial Peptides Human beta-Defensins Stimulate Epidermal Keratinocyte Migration, Proliferation and Production of Proinflammatory Cytokines and Chemokines. *J Invest Dermatol* **127**, 594-604.
237. Funderburg, N., Lederman, M.M., Feng, Z., Drage, M.G., Jadlowsky, J., Harding, C.V., Weinberg, A., Sieg, S.F. (2007) Human -defensin-3 activates professional antigen-presenting cells via Toll-like receptors 1 and 2. *Proc Natl Acad Sci U S A* **104**, 18631-5.
238. Lai, Y., Adhikarakunnathu, S., Bhardwaj, K., Ranjith-Kumar, C.T., Wen, Y., Jordan, J.L., Wu, L.H., Dragnea, B., San Mateo, L., Kao, C.C. (2011) LL37 and cationic peptides enhance TLR3 signaling by viral double-stranded RNAs. *PLoS One* **6**, e26632.

239. Davey, G.M., Wojtasiak, M., Proietto, A.I., Carbone, F.R., Heath, W.R., Bedoui, S. (2010) Cutting edge: priming of CD8 T cell immunity to herpes simplex virus type 1 requires cognate TLR3 expression in vivo. *J Immunol* **184**, 2243-6.
240. Steinman, R.M., Turley, S., Mellman, I., Inaba, K. (2000) The induction of tolerance by dendritic cells that have captured apoptotic cells. *J Exp Med* **191**, 411-6.
241. Gurunathan, S., Klinman, D.M., Seder, R.A. (2000) DNA vaccines: immunology, application, and optimization*. *Annu Rev Immunol* **18**, 927-74.
242. Davis, H.L., McCluskie, M.J. (1999) DNA vaccines for viral diseases. *Microbes Infect* **1**, 7-21.
243. Babiuk, L.A., van Drunen Littel-van den, H., Babiuk, S.L. (1999) Immunization of animals: from DNA to the dinner plate. *Vet Immunol Immunopathol* **72**, 189-202.
244. Van Drunen Littel-van den Hurk, S., Braun, R.P., Lewis, P.J., Karvonen, B.C., Babiuk, L.A., Griebel, P.J. (1999) Immunization of neonates with DNA encoding a bovine herpesvirus glycoprotein is effective in the presence of maternal antibodies. *Viral Immunol* **12**, 67-77.
245. Ulmer, J.B., Donnelly, J.J., Parker, S.E., Rhodes, G.H., Felgner, P.L., Dwarki, V.J., Gromkowski, S.H., Deck, R.R., DeWitt, C.M., Friedman, A., et al. (1993) Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* **259**, 1745-9.
246. Donnelly, J.J., Martinez, D., Jansen, K.U., Ellis, R.W., Montgomery, D.L., Liu, M.A. (1996) Protection against papillomavirus with a polynucleotide vaccine. *J Infect Dis* **173**, 314-20.
247. Wolff, J.A., Malone, R.W., Williams, P., Chong, W., Acsadi, G., Jani, A., Felgner, P.L. (1990) Direct gene transfer into mouse muscle in vivo. *Science* **247**, 1465-8.
248. Tang, D.C., DeVit, M., Johnston, S.A. (1992) Genetic immunization is a simple method for eliciting an immune response. *Nature* **356**, 152-4.
249. Babiuk, L.A., van Drunen Littel-van den Hurk, S., Loehr, B.I., Uwiera, R. (2000) Veterinary applications of DNA vaccines. *Dev Biol (Basel)* **104**, 73-81.
250. Babiuk, L.A., Lewis, J., van den Hurk, S., Braun, R. (1999) DNA immunization: present and future. *Adv Vet Med* **41**, 163-79.
251. van Drunen Littel-van den Hurk, S., Loehr, B.I., Babiuk, L.A. (2001) Immunization of livestock with DNA vaccines: current studies and future prospects. *Vaccine* **19**, 2474-9.
252. Ulmer, J.B., Wahren, B., Liu, M.A. (2006) Gene-based vaccines: recent technical and clinical advances. *Trends Mol Med* **12**, 216-22.
253. Boyle, J.S., Koniaras, C., Lew, A.M. (1997) Influence of cellular location of expressed antigen on the efficacy of DNA vaccination: cytotoxic T lymphocyte and antibody responses are suboptimal when antigen is cytoplasmic after intramuscular DNA immunization. *Int Immunol* **9**, 1897-906.
254. Drew, D.R., Lightowlers, M., Strugnell, R.A. (2000) Humoral immune responses to DNA vaccines expressing secreted, membrane bound and non-secreted forms of the Tania ovis 45W antigen. *Vaccine* **18**, 2522-32.
255. Dyer, C.M., Zhan, Y., Brady, J.L., Carbone, F.R., Smyth, M.J., Lew, A.M. (2004) Unexpectedly, induction of cytotoxic T lymphocytes enhances the humoral response after DNA immunization. *Blood* **103**, 3073-5.
256. Roy, M.J., Wu, M.S., Barr, L.J., Fuller, J.T., Tussey, L.G., Speller, S., Culp, J., Burkholder, J.K., Swain, W.F., Dixon, R.M., Widera, G., Vessey, R., King, A., Ogg, G.,

Gallimore, A., Haynes, J.R., Heydenburg Fuller, D. (2000) Induction of antigen-specific CD8+ T cells, T helper cells, and protective levels of antibody in humans by particle-mediated administration of a hepatitis B virus DNA vaccine. *Vaccine* **19**, 764-78.

257. Justewicz, D.M., Webster, R.G. (1996) Long-term maintenance of B cell immunity to influenza virus hemagglutinin in mice following DNA-based immunization. *Virology* **224**, 10-7.
258. Deck, R.R., DeWitt, C.M., Donnelly, J.J., Liu, M.A., Ulmer, J.B. (1997) Characterization of humoral immune responses induced by an influenza hemagglutinin DNA vaccine. *Vaccine* **15**, 71-8.
259. Kang, Y., Calvo, P.A., Daly, T.M., Long, C.A. (1998) Comparison of humoral immune responses elicited by DNA and protein vaccines based on merozoite surface protein-1 from Plasmodium yoelii, a rodent malaria parasite. *J Immunol* **161**, 4211-9.
260. Feltquate, D.M., Heaney, S., Webster, R.G., Robinson, H.L. (1997) Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. *J Immunol* **158**, 2278-84.
261. Leitner, W.W., Seguin, M.C., Ballou, W.R., Seitz, J.P., Schultz, A.M., Sheehy, M.J., Lyon, J.A. (1997) Immune responses induced by intramuscular or gene gun injection of protective deoxyribonucleic acid vaccines that express the circumsporozoite protein from Plasmodium berghei malaria parasites. *J Immunol* **159**, 6112-9.
262. Torres, C.A., Iwasaki, A., Barber, B.H., Robinson, H.L. (1997) Differential dependence on target site tissue for gene gun and intramuscular DNA immunizations. *J Immunol* **158**, 4529-32.
263. Braun, R.P., Babiuk, L.A., Loehr, B.I., van Drunen Littel-van den, H. (1999) Particle-mediated DNA immunization of cattle confers long-lasting immunity against bovine herpesvirus-1. *Virology* **265**, 46-56.
264. Loehr, B.I., Pontarollo, R., Rankin, R., Latimer, L., Willson, P., Babiuk, L.A., van Drunen Littel-van den Hurk, S. (2001) Priming by DNA immunization augments T-cell responses induced by modified live bovine herpesvirus vaccine. *J Gen Virol* **82**, 3035-43.
265. Loehr, B.I., Willson, P., Babiuk, L.A., van Drunen Littel-van den, H. (2000) Gene gun-mediated DNA immunization primes development of mucosal immunity against bovine herpesvirus 1 in cattle. *J Virol* **74**, 6077-86.
266. Corr, M., Lee, D.J., Carson, D.A., Tighe, H. (1996) Gene vaccination with naked plasmid DNA: mechanism of CTL priming. *J Exp Med* **184**, 1555-60.
267. Doe, B., Selby, M., Barnett, S., Baenziger, J., Walker, C.M. (1996) Induction of cytotoxic T lymphocytes by intramuscular immunization with plasmid DNA is facilitated by bone marrow-derived cells. *Proc Natl Acad Sci U S A* **93**, 8578-83.
268. Cho, J.H., Youn, J.W., Sung, Y.C. (2001) Cross-priming as a predominant mechanism for inducing CD8(+) T cell responses in gene gun DNA immunization. *J Immunol* **167**, 5549-57.
269. Casares, S., Inaba, K., Brumeanu, T.D., Steinman, R.M., Bona, C.A. (1997) Antigen presentation by dendritic cells after immunization with DNA encoding a major histocompatibility complex class II-restricted viral epitope. *J Exp Med* **186**, 1481-6.
270. Whitton, J.L., Zhang, J. (1995) Principles of cytotoxic T lymphocyte induction and recognition. *Curr Top Microbiol Immunol* **202**, 247-59.

271. Porgador, A., Irvine, K.R., Iwasaki, A., Barber, B.H., Restifo, N.P., Germain, R.N. (1998) Predominant role for directly transfected dendritic cells in antigen presentation to CD8+ T cells after gene gun immunization. *J Exp Med* **188**, 1075-82.
272. Fonteneau, J.F., Kavanagh, D.G., Lirvall, M., Sanders, C., Cover, T.L., Bhardwaj, N., Larsson, M. (2003) Characterization of the MHC class I cross-presentation pathway for cell-associated antigens by human dendritic cells. *Blood* **102**, 4448-55.
273. Kleijmeer, M.J., Escola, J.M., UytdeHaag, F.G., Jakobson, E., Griffith, J.M., Osterhaus, A.D., Stoorvogel, W., Melief, C.J., Rabouille, C., Geuze, H.J. (2001) Antigen loading of MHC class I molecules in the endocytic tract. *Traffic* **2**, 124-37.
274. Wakim, L.M., Bevan, M.J. (2011) Cross-dressed dendritic cells drive memory CD8+ T-cell activation after viral infection. *Nature* **471**, 629-32.
275. Condon, C., Watkins, S.C., Celluzzi, C.M., Thompson, K., Falo, L.D., Jr. (1996) DNA-based immunization by in vivo transfection of dendritic cells. *Nat Med* **2**, 1122-8.
276. van Donkersgoed, J., Klassen, P. (1995) Serological study of a modified-live virus IBR vaccine given to feedlot calves after arrival. *Can Vet J* **36**, 394.
277. Castrucci, G., Frigeri, F., Salvatori, D., Ferrari, M., Dico, M.L., Rotola, A., Sardonini, Q., Petrini, S., Cassai, E. (2002) A study on latency in calves by five vaccines against bovine herpesvirus-1 infection. *Comp Immunol Microbiol Infect Dis* **25**, 205-15.
278. van Donkersgoed, J. (1991) Diagnosing and managing the respiratory form of infectious bovine rhinotracheitis. *Veterinary Medicine* **1**, 86.
279. Radostits, O.M., et. al. (2000) Bovine Herpesviruses. In *Veterinary Medicine: a textbook of the diseases of cattle, sheep, pigs, goats and horses* (O. M. Radostits, ed) W. B. Saunders Co. Ltd., New York, NY. 1173.
280. Manickan, E., Kanangat, S., Rouse, R.J., Yu, Z., Rouse, B.T. (1997) Enhancement of immune response to naked DNA vaccine by immunization with transfected dendritic cells. *J Leukoc Biol* **61**, 125-32.
281. Steinman, R.M., Hemmi, H. (2006) Dendritic cells: translating innate to adaptive immunity. *Curr Top Microbiol Immunol* **311**, 17-58.
282. Pantel, A., Cheong, C., Dandamudi, D., Shrestha, E., Mehandru, S., Brane, L., Ruane, D., Teixeira, A., Bozzacco, L., Steinman, R.M., Longhi, M.P. (2012) A new synthetic TLR4 agonist, GLA, allows dendritic cells targeted with antigen to elicit Th1 T-cell immunity in vivo. *Eur J Immunol* **42**, 101-9.
283. Steinman, R.M. (2012) Decisions about dendritic cells: past, present, and future. *Annu Rev Immunol* **30**, 1-22.
284. Desmet, C.J., Ishii, K.J. (2012) Nucleic acid sensing at the interface between innate and adaptive immunity in vaccination. *Nat Rev Immunol* **12**, 479-91.
285. Sparwasser, T., Koch, E.S., Vabulas, R.M., Heeg, K., Lipford, G.B., Ellwart, J.W., Wagner, H. (1998) Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur J Immunol* **28**, 2045-54.
286. Bachmann, M.F., Kopf, M., Marsland, B.J. (2006) Chemokines: more than just road signs. *Nat Rev Immunol* **6**, 159-64.
287. Hoover, D.M., Chertov, O., Lubkowski, J. (2001) The structure of human beta-defensin-1: new insights into structural properties of beta-defensins. *J Biol Chem* **276**, 39021-6.
288. Bell, D., Young, J.W., Banchereau, J. (1999) Dendritic cells. *Adv Immunol* **72**, 255-324.
289. Caminschi, I., Lahoud, M.H., Shortman, K. (2009) Enhancing immune responses by targeting antigen to DC. *Eur J Immunol* **39**, 931-8.

290. Sallusto, F., Schaerli, P., Loetscher, P., Schaniel, C., Lenig, D., Mackay, C.R., Qin, S., Lanzavecchia, A. (1998) Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur J Immunol* **28**, 2760-9.
291. Dieu, M.C., Vanbervliet, B., Vicari, A., Bridon, J.M., Oldham, E., Ait-Yahia, S., Briere, F., Zlotnik, A., Lebecque, S., Caux, C. (1998) Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J Exp Med* **188**, 373-86.
292. Tacken, P.J., Torensma, R., Figdor, C.G. (2006) Targeting antigens to dendritic cells in vivo. *Immunobiology* **211**, 599-608.
293. Presicce, P., Giannelli, S., Taddeo, A., Villa, M.L., Della Bella, S. (2009) Human defensins activate monocyte-derived dendritic cells, promote the production of proinflammatory cytokines, and up-regulate the surface expression of CD91. *J Leukoc Biol* **86**, 941-8.
294. Rohrl, J., Yang, D., Oppenheim, J.J., Hehlgans, T. (2008) Identification and Biological Characterization of Mouse beta-defensin 14, the orthologue of human beta-defensin 3. *J Biol Chem* **283**, 5414-9.
295. Rohrl, J., Yang, D., Oppenheim, J.J., Hehlgans, T. (2010) Specific binding and chemotactic activity of mBD4 and its functional orthologue hBD2 to CCR6-expressing cells. *J Biol Chem* **285**, 7028-34.
296. Sallusto, F., Palermo, B., Lenig, D., Miettinen, M., Matikainen, S., Julkunen, I., Forster, R., Burgstahler, R., Lipp, M., Lanzavecchia, A. (1999) Distinct patterns and kinetics of chemokine production regulate dendritic cell function. *Eur J Immunol* **29**, 1617-25.
297. Morgera, F., Pacor, S., Creatti, L., Antcheva, N., Vaccari, L., Tossi, A. (2011) Effects on APC antigen presenting cells of short-term interaction with the human human host defense peptide beta-defensin 2. *Biochem J* **436**, 537-46.
298. Vanbervliet, B., Homey, B., Durand, I., Massacrier, C., Ait-Yahia, S., de Bouteiller, O., Vicari, A., Caux, C. (2002) Sequential involvement of CCR2 and CCR6 ligands for immature dendritic cell recruitment: possible role at inflamed epithelial surfaces. *Eur J Immunol* **32**, 231-42.
299. Stebbing, J., Savage, P., Patterson, S., Gazzard, B. (2004) All for CD91 and CD91 for all. *J Antimicrob Chemother* **53**, 1-3.
300. Oppenheim, J.J., Yang, D. (2005) Alarmins: chemotactic activators of immune responses. *Curr Opin Immunol* **17**, 359-65.
301. Elahi, S., Buchanan, R.M., Attah-Poku, S., Townsend, H.G., Babiuk, L.A., Gerdts, V. (2006) The host defense peptide beta-defensin 1 confers protection against Bordetella pertussis in newborn piglets. *Infect Immun* **74**, 2338-52.
302. Hiratsuka, T., Nakazato, M., Date, Y., Ashitani, J., Minematsu, T., Chino, N., Matsukura, S. (1998) Identification of human beta-defensin-2 in respiratory tract and plasma and its increase in bacterial pneumonia. *Biochem Biophys Res Commun* **249**, 943-7.
303. Zasloff, M. (1987) Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proc Natl Acad Sci U S A* **84**, 5449-53.
304. Good, L., Nielsen, P.E. (1998) Inhibition of translation and bacterial growth by peptide nucleic acid targeted to ribosomal RNA. *Proc Natl Acad Sci U S A* **95**, 2073-6.

305. Shen, Y., Liu, J., Estiu, G., Isin, B., Ahn, Y.Y., Lee, D.S., Barabasi, A.L., Kapatral, V., Wiest, O., Oltvai, Z.N. (2010) Blueprint for antimicrobial hit discovery targeting metabolic networks. *Proc Natl Acad Sci U S A* **107**, 1082-7.
306. Garcia, J.R., Krause, A., Schulz, S., Rodriguez-Jimenez, F.J., Kluver, E., Adermann, K., Forssmann, U., Frimpong-Boateng, A., Bals, R., Forssmann, W.G. (2001) Human beta-defensin 4: a novel inducible peptide with a specific salt-sensitive spectrum of antimicrobial activity. *Faseb J* **15**, 1819-21.
307. Norimatsu, M., Harris, J., Chance, V., Dougan, G., Howard, C.J., Villarreal-Ramos, B. (2003) Differential response of bovine monocyte-derived macrophages and dendritic cells to infection with Salmonella typhimurium in a low-dose model in vitro. *Immunology* **108**, 55-61.
308. Howard, C.J., Sopp, P., Brownlie, J., Kwong, L.S., Parsons, K.R., Taylor, G. (1997) Identification of two distinct populations of dendritic cells in afferent lymph that vary in their ability to stimulate T cells. *J Immunol* **159**, 5372-82.
309. Werling, D., Hope, J.C., Chaplin, P., Collins, R.A., Taylor, G., Howard, C.J. (1999) Involvement of caveolae in the uptake of respiratory syncytial virus antigen by dendritic cells. *J Leukoc Biol* **66**, 50-8.
310. van Drunen Littel-van den Hurk, S., Van Donkersgoed, J., Kowalski, J., van den Hurk, J.V., Harland, R., Babiuk, L.A., Zamb, T.J. (1994) A subunit gIV vaccine, produced by transfected mammalian cells in culture, induces mucosal immunity against bovine herpesvirus-1 in cattle. *Vaccine* **12**, 1295-302.
311. Manoj, S., Griebel, P.J., Babiuk, L.A., van Drunen Littel-van den Hurk, S. (2004) Modulation of immune responses to bovine herpesvirus-1 in cattle by immunization with a DNA vaccine encoding glycoprotein D as a fusion protein with bovine CD154. *Immunology* **112**, 328-38.
312. Glew, E.J., Carr, B.V., Brackenbury, L.S., Hope, J.C., Charleston, B., Howard, C.J. (2003) Differential effects of bovine viral diarrhoea virus on monocytes and dendritic cells. *J Gen Virol* **84**, 1771-80.
313. Huang, Y., Babiuk, L.A., van Drunen Littel-van den Hurk, S. (2005) Immunization with a bovine herpesvirus 1 glycoprotein B DNA vaccine induces cytotoxic T-lymphocyte responses in mice and cattle. *J Gen Virol* **86**, 887-98.
314. Frevert, C.W., Wong, V.A., Goodman, R.B., Goodwin, R., Martin, T.R. (1998) Rapid fluorescence-based measurement of neutrophil migration in vitro. *J Immunol Methods* **213**, 41-52.
315. Yang, D., Chen, Q., Chertov, O., Oppenheim, J.J. (2000) Human neutrophil defensins selectively chemoattract naive T and immature dendritic cells. *J Leukoc Biol* **68**, 9-14.
316. Nagase, H., Yamaguchi, M., Jibiki, S., Yamada, H., Ohta, K., Kawasaki, H., Yoshie, O., Yamamoto, K., Morita, Y., Hirai, K. (1999) Eosinophil chemotaxis by chemokines: a study by a simple photometric assay. *Allergy* **54**, 944-50.
317. Falk, W., Goodwin, R.H., Jr., Leonard, E.J. (1980) A 48-well micro chemotaxis assembly for rapid and accurate measurement of leukocyte migration. *J Immunol Methods* **33**, 239-47.
318. Gliddon, D.R., Hope, J.C., Brooke, G.P., Howard, C.J. (2004) DEC-205 expression on migrating dendritic cells in afferent lymph. *Immunology* **111**, 262-72.

319. Landi, A., Babiuk, L.A., van Drunen Littel-van den Hurk, S. (2007) High transfection efficiency, gene expression, and viability of monocyte-derived human dendritic cells after nonviral gene transfer. *J Leukoc Biol* **82**, 849-60.
320. Riegler, S., Hebart, H., Einsele, H., Brossart, P., Jahn, G., Sinzger, C. (2000) Monocyte-derived dendritic cells are permissive to the complete replicative cycle of human cytomegalovirus. *J Gen Virol* **81**, 393-9.
321. Howard, C.J., Brooke, G.P., Werling, D., Sopp, P., Hope, J.C., Parsons, K.R., Collins, R.A. (1999) Dendritic cells in cattle: phenotype and function. *Vet Immunol Immunopathol* **72**, 119-24.
322. Howard, C.J., Hope, J.C., Villarreal-Ramos, B. (2004) Contribution of in vivo and ex vivo studies to understanding the role of antigen-presenting cells and T cell subsets in immunity to cattle diseases. *Anim Health Res Rev* **5**, 1-13.
323. Howard, C.J., Sopp, P., Brownlie, J., Parsons, K.R., Kwong, L.S., Collins, R.A. (1996) Afferent lymph veiled cells stimulate proliferative responses in allogeneic CD4+ and CD8+ T cells but not gamma delta TCR+ T cells. *Immunology* **88**, 558-64.
324. Mirkovitch, J., Konig, A., Sauter, K.S., Brcic, M., Hope, J.C., Howard, C.J., Jungi, T.W. (2006) Single-cell analysis divides bovine monocyte-derived dendritic cells into subsets expressing either high or low levels of inducible nitric oxide synthase. *Vet Immunol Immunopathol* **114**, 1-14.
325. MacHugh, N.D., Bensaid, A., Davis, W.C., Howard, C.J., Parsons, K.R., Jones, B., Kaushal, A. (1988) Characterization of a bovine thymic differentiation antigen analogous to CD1 in the human. *Scand J Immunol* **27**, 541-7.
326. Cella, M., Sallusto, F., Lanzavecchia, A. (1997) Origin, maturation and antigen presenting function of dendritic cells. *Curr Opin Immunol* **9**, 10-6.
327. Gliddon, D.R., Howard, C.J. (2002) CD26 is expressed on a restricted subpopulation of dendritic cells in vivo. *Eur J Immunol* **32**, 1472-81.
328. Howard, C.J., Charleston, B., Stephens, S.A., Sopp, P., Hope, J.C. (2004) The role of dendritic cells in shaping the immune response. *Anim Health Res Rev* **5**, 191-5.
329. Howard, C.J., Collins, R.A., Sopp, P., Brooke, G.P., Kwong, L.S., Parsons, K.R., Weynants, V., Letesson, J.J., Bembridge, G.P. (1999) T-cell responses and the influence of dendritic cells in cattle. *Adv Vet Med* **41**, 275-88.
330. Hope, J.C., Whelan, A.O., Hewinson, R.G., Vordermeier, M., Howard, C.J. (2003) Maturation of bovine dendritic cells by lipopeptides. *Vet Immunol Immunopathol* **95**, 21-31.
331. Norimatsu, M., Chance, V., Dougan, G., Howard, C.J., Villarreal-Ramos, B. (2004) Live Salmonella enterica serovar Typhimurium (S. Typhimurium) elicit dendritic cell responses that differ from those induced by killed S. Typhimurium. *Vet Immunol Immunopathol* **98**, 193-201.
332. Werling, D., Collins, R.A., Taylor, G., Howard, C.J. (2002) Cytokine responses of bovine dendritic cells and T cells following exposure to live or inactivated bovine respiratory syncytial virus. *J Leukoc Biol* **72**, 297-304.
333. Bauer, F., Schweimer, K., Kluver, E., Conejo-Garcia, J.R., Forssmann, W.G., Rosch, P., Adermann, K., Sticht, H. (2001) Structure determination of human and murine beta-defensins reveals structural conservation in the absence of significant sequence similarity. *Protein Sci* **10**, 2470-9.

334. Wu, Z., Hoover, D.M., Yang, D., Boulegue, C., Santamaria, F., Oppenheim, J.J., Lubkowski, J., Lu, W. (2003) Engineering disulfide bridges to dissect antimicrobial and chemotactic activities of human beta-defensin 3. *Proc Natl Acad Sci U S A* **100**, 8880-5.
335. Ahmed, A.K., Schaffer, S.W., Wetlaufer, D.B. (1975) Nonenzymic reactivation of reduced bovine pancreatic ribonuclease by air oxidation and by glutathione oxidoreduction buffers. *J Biol Chem* **250**, 8477-82.
336. Ferrer, M., Woodward, C., Barany, G. (1992) Solid-phase synthesis of bovine pancreatic trypsin inhibitor (BPTI) and two analogues. A chemical approach for evaluating the role of disulfide bridges in protein folding and stability. *Int J Pept Protein Res* **40**, 194-207.
337. Tuting, T. (1999) The immunology of cutaneous DNA immunization. *Curr Opin Mol Ther* **1**, 216-25.
338. Mwangi, W., Brown, W.C., Splitter, G.A., Zhuang, Y., Kegerreis, K., Palmer, G.H. (2005) Enhancement of antigen acquisition by dendritic cells and MHC class II-restricted epitope presentation to CD4+ T cells using VP22 DNA vaccine vectors that promote intercellular spreading following initial transfection. *J Leukoc Biol* **78**, 401-11.
339. Zhuang, Y., Mwangi, W., Brown, W.C., Davis, W.C., Hope, J.C., Palmer, G.H. (2006) Characterization of a phenotypically unique population of CD13+ dendritic cells resident in the spleen. *Clin Vaccine Immunol* **13**, 1064-9.
340. Pinchuk, L.M., Boyd, B.L., Kruger, E.F., Roditi, I., Furger, A. (2003) Bovine dendritic cells generated from monocytes and bone marrow progenitors regulate immunoglobulin production in peripheral blood B cells. *Comp Immunol Microbiol Infect Dis* **26**, 233-49.
341. Mwangi, W., Brown, W.C., Splitter, G.A., Davies, C.J., Howard, C.J., Hope, J.C., Aida, Y., Zhuang, Y., Hunter, B.J., Palmer, G.H. (2007) DNA vaccine construct incorporating intercellular trafficking and intracellular targeting motifs effectively primes and induces memory B- and T-cell responses in outbred animals. *Clin Vaccine Immunol* **14**, 304-11.
342. Mena, A., Nichani, A.K., Popowych, Y., Ioannou, X.P., Godson, D.L., Mutwiri, G.K., Hecker, R., Babiuk, L.A., Griebel, P. (2003) Bovine and ovine blood mononuclear leukocytes differ markedly in innate immune responses induced by Class A and Class B CpG-oligodeoxynucleotide. *Oligonucleotides* **13**, 245-59.
343. Wattegedera, S., Sills, K., Howard, C.J., Hope, J.C., McInnes, C.J., Entrican, G. (2004) Variability in cytokine production and cell proliferation by mitogen-activated ovine peripheral blood mononuclear cells: modulation by interleukin (IL)-10 and IL-12. *Vet Immunol Immunopathol* **102**, 67-76.
344. Mwangi, W., Brown, W.C., Lewin, H.A., Howard, C.J., Hope, J.C., Baszler, T.V., Caplazi, P., Abbott, J., Palmer, G.H. (2002) DNA-encoded fetal liver tyrosine kinase 3 ligand and granulocyte macrophage-colony-stimulating factor increase dendritic cell recruitment to the inoculation site and enhance antigen-specific CD4+ T cell responses induced by DNA vaccination of outbred animals. *J Immunol* **169**, 3837-46.
345. Dhople, V., Krukemeyer, A., Ramamoorthy, A. (2006) The human beta-defensin-3, an antibacterial peptide with multiple biological functions. *Biochim Biophys Acta* **1758**, 1499-512.
346. Kluver, E., Adermann, K., Schulz, A. (2006) Synthesis and structure-activity relationship of beta-defensins, multi-functional peptides of the immune system. *J Pept Sci* **12**, 243-57.
347. Routsias, J.G., Karagounis, P., Parvulesku, G., Legakis, N.J., Tsakris, A. (2010) In vitro bactericidal activity of human beta-defensin 2 against nosocomial strains. *Peptides* **31**, 1654-60.

348. Taylor, K., Barran, P.E., Dorin, J.R. (2008) Structure-activity relationships in beta-defensin peptides. *Biopolymers* **90**, 1-7.
349. Scudiero, O., Galdiero, S., Cantisani, M., Di Noto, R., Vitiello, M., Galdiero, M., Naclerio, G., Cassiman, J.J., Pedone, C., Castaldo, G., Salvatore, F. (2010) Novel synthetic, salt-resistant analogs of human beta-defensins 1 and 3 endowed with enhanced antimicrobial activity. *Antimicrob Agents Chemother* **54**, 2312-22.
350. Hoover, D.M., Wu, Z., Tucker, K., Lu, W., Lubkowski, J. (2003) Antimicrobial characterization of human beta-defensin 3 derivatives. *Antimicrob Agents Chemother* **47**, 2804-9.
351. Kluver, E., Schulz-Maronde, S., Scheid, S., Meyer, B., Forssmann, W.G., Adermann, K. (2005) Structure-activity relation of human beta-defensin 3: influence of disulfide bonds and cysteine substitution on antimicrobial activity and cytotoxicity. *Biochemistry* **44**, 9804-16.
352. Circo, R., Skerlavaj, B., Gennaro, R., Amoroso, A., Zanetti, M. (2002) Structural and functional characterization of hBD-1(Ser35), a peptide deduced from a DEFB1 polymorphism. *Biochem Biophys Res Commun* **293**, 586-92.
353. Taylor, K., McCullough, B., Clarke, D.J., Langley, R.J., Pechenick, T., Hill, A., Campopiano, D.J., Barran, P.E., Dorin, J.R., Govan, J.R. (2007) Covalent dimer species of beta-defensin Defr1 display potent antimicrobial activity against multidrug-resistant bacterial pathogens. *Antimicrob Agents Chemother* **51**, 1719-24.
354. Harder, J., Bartels, J., Christophers, E., Schroder, J.M. (2001) Isolation and characterization of human beta -defensin-3, a novel human inducible peptide antibiotic. *J Biol Chem* **276**, 5707-13.
355. Kluver, E., Schulz, A., Forssmann, W.G., Adermann, K. (2002) Chemical synthesis of beta-defensins and LEAP-1/hepcidin. *J Pept Res* **59**, 241-8.
356. Schulz, A., Kluver, E., Schulz-Maronde, S., Adermann, K. (2005) Engineering disulfide bonds of the novel human beta-defensins hBD-27 and hBD-28: differences in disulfide formation and biological activity among human beta-defensins. *Biopolymers* **80**, 34-49.
357. Krishnakumari, V., Sharadadevi, A., Singh, S., Nagaraj, R. (2003) Single disulfide and linear analogues corresponding to the carboxy-terminal segment of bovine beta-defensin-2: effects of introducing the beta-hairpin nucleating sequence d-pro-gly on antibacterial activity and Biophysical properties. *Biochemistry* **42**, 9307-15.
358. Mandal, M., Jagannadham, M.V., Nagaraj, R. (2002) Antibacterial activities and conformations of bovine beta-defensin BNBD-12 and analogs:structural and disulfide bridge requirements for activity. *Peptides* **23**, 413-8.
359. Boniotto, M., Antcheva, N., Zelezetsky, I., Tossi, A., Palumbo, V., Verga Falzacappa, M.V., Sgubin, S., Braida, L., Amoroso, A., Crovella, S. (2003) A study of host defence peptide beta-defensin 3 in primates. *Biochem J* **374**, 707-14.
360. Bensch, K.W., Raida, M., Magert, H.J., Schulz-Knappe, P., Forssmann, W.G. (1995) hBD-1: a novel beta-defensin from human plasma. *FEBS Lett* **368**, 331-5.
361. Pazgier, M., Prahl, A., Hoover, D.M., Lubkowski, J. (2007) Studies of the biological properties of human beta -defensin 1. *J Biol Chem* **282**, 1819-29.
362. Antcheva, N., Morgera, F., Creatti, L., Vaccari, L., Pag, U., Pacor, S., Shai, Y., Sahl, H.G., Tossi, A. (2009) Artificial beta-defensin based on a minimal defensin template. *Biochem J* **421**, 435-47.

363. Zhao, C., Nguyen, T., Liu, L., Sacco, R.E., Brogden, K.A., Lehrer, R.I. (2001) Gallinacin-3, an inducible epithelial beta-defensin in the chicken. *Infect Immun* **69**, 2684-91.
364. de Leeuw, E., Rajabi, M., Zou, G., Pazgier, M., Lu, W. (2009) Selective arginines are important for the antibacterial activity and host cell interaction of human alpha-defensin 5. *FEBS Lett* **583**, 2507-12.
365. Wakefield, J.S., Gale, J.S., Berridge, M.V., Jordan, T.W., Ford, H.C. (1982) Is Percoll innocuous to cells? *Biochem J* **202**, 795-7.
366. Graziani-Bowering, G.M., Graham, J.M., Filion, L.G. (1997) A quick, easy and inexpensive method for the isolation of human peripheral blood monocytes. *J Immunol Methods* **207**, 157-68.
367. Taylor, K., Clarke, D.J., McCullough, B., Chin, W., Seo, E., Yang, D., Oppenheim, J., Uhrin, D., Govan, J.R., Campopiano, D.J., MacMillan, D., Barran, P., Dorin, J.R. (2008) Analysis and separation of residues important for the chemoattractant and antimicrobial activities of beta-defensin 3. *J Biol Chem* **283**, 6631-9.
368. Struyf, S., Proost, P., Lenaerts, J.P., Stoops, G., Wuyts, A., Van Damme, J. (2001) Identification of a blood-derived chemoattractant for neutrophils and lymphocytes as a novel CC chemokine, Regakine-1. *Blood* **97**, 2197-204.
369. Caux, C., Vanbervliet, B., Massacrier, C., Ait-Yahia, S., Vaure, C., Chemin, K., Dieu, N., Mc, Vicari, A. (2002) Regulation of dendritic cell recruitment by chemokines. *Transplantation* **73**, S7-11.
370. Wilson, N.S., Young, L.J., Kupresanin, F., Naik, S.H., Vremec, D., Heath, W.R., Akira, S., Shortman, K., Boyle, J., Maraskovsky, E., Belz, G.T., Villadangos, J.A. (2007) Normal proportion and expression of maturation markers in migratory dendritic cells in the absence of germs or Toll-like receptor signaling. *Immunol Cell Biol*.
371. Dutia, B.M., Ross, A.J., Hopkins, J. (1993) Analysis of the monoclonal antibodies comprising WC6. *Vet Immunol Immunopathol* **39**, 193-9.
372. McKeever, D.J., MacHugh, N.D., Goddeeris, B.M., Awino, E., Morrison, W.I. (1991) Bovine afferent lymph veiled cells differ from blood monocytes in phenotype and accessory function. *J Immunol* **147**, 3703-9.
373. Le Borgne, M., Dubois, B., Kaiserlian, D. (2007) [Dendritic cells of mucosa and skin: "recruited for vaccination"]. *Med Sci (Paris)* **23**, 819-25.
374. Bowland, S.L., Shewen, P.E. (2000) Bovine respiratory disease: commercial vaccines currently available in Canada. *Can Vet J* **41**, 33-48.
375. Snowder, G.D., Van Vleck, L.D., Cundiff, L.V., Bennett, G.L. (2006) Bovine respiratory disease in feedlot cattle: environmental, genetic, and economic factors. *J Anim Sci* **84**, 1999-2008.
376. Kahrs, R.F. (1981) Viral disease of cattle. Iowa State University Press, Ames, Iowa 135-136.
377. Raaperi, K., Bougeard, S., Aleksejev, A., Orro, T., Viltrop, A. (2012) Association of herd BRSV and BHV-1 seroprevalence with respiratory disease and reproductive performance in adult dairy cattle. *Acta Vet Scand* **54**, 4.
378. van Drunen Littel-van den Hurk, S., Parker, M.D., Massie, B., van den Hurk, J.V., Harland, R., Babiuk, L.A., Zamb, T.J. (1993) Protection of cattle from BHV-1 infection by immunization with recombinant glycoprotein gIV. *Vaccine* **11**, 25-35.

379. Wyler, R., Engels, M., Schwyzer, M. (1989) Infectious bovine rhinotracheitis/vulvovaginitis (BHV-1). In *Herpesvirus disease of cattle, horses and pigs. Developments in veterinary virology.* (G. Wittman, ed) Kluwer Academic Publishers, Boston 1-72.
380. Zimmerman, A.D., Buterbaugh, R.E., Herbert, J.M., Hass, J.M., Frank, N.E., Luempert Iii, L.G., Chase, C.C. (2007) Efficacy of bovine herpesvirus-1 inactivated vaccine against abortion and stillbirth in pregnant heifers. *J Am Vet Med Assoc* **231**, 1386-9.
381. Pospisil, Z., Krejci, J., Jinek, P., Lany, P., Zendulkova, D., Cihal, P. (1996) Development of a disease control programme based on the use of an inactivated vaccine against infectious bovine rhinotracheitis. *Vet Microbiol* **53**, 199-206.
382. Meeusen, E.N., Walker, J., Peters, A., Pastoret, P.P., Jungersen, G. (2007) Current status of veterinary vaccines. *Clin Microbiol Rev* **20**, 489-510, table of contents.
383. van Drunen Littel-van den Hurk, S., Myers, D., Doig, P.A., Karvonen, B., Habermehl, M., Babiuk, L.A., Jelinski, M., Van Donkersgoed, J., Schlesinger, K., Rinehart, C. (2001) Identification of a mutant bovine herpesvirus-1 (BHV-1) in post-arrival outbreaks of IBR in feedlot calves and protection with conventional vaccination. *Can J Vet Res* **65**, 81-8.
384. Lowe, D.B., Shearer, M.H., Kennedy, R.C. (2006) DNA vaccines: successes and limitations in cancer and infectious disease. *J Cell Biochem* **98**, 235-42.
385. Kutzler, M.A., Weiner, D.B. (2008) DNA vaccines: ready for prime time? *Nat Rev Genet* **9**, 776-88.
386. Kutzler, M.A., Weiner, D.B. (2004) Developing DNA vaccines that call to dendritic cells. *J Clin Invest* **114**, 1241-4.
387. Ivory, C., Chadee, K. (2004) DNA vaccines: designing strategies against parasitic infections. *Genet Vaccines Ther* **2**, 17.
388. Zhang, H.H., Yang, X.M., Xie, Q.M., Ma, J.Y., Luo, Y.N., Cao, Y.C., Chen, F., Bi, Y.Z. (2010) The potent adjuvant effects of chicken beta-defensin-1 when genetically fused with infectious bursal disease virus VP2 gene. *Vet Immunol Immunopathol* **136**, 92-7.
389. Mackenzie-Dyck, S., Attah-Poku, S., Juillard, V., Babiuk, L.A., van Drunen Littel-van den Hurk, S. (2011) The synthetic peptides bovine enteric beta-defensin (EBD), bovine neutrophil beta-defensin (BNBD) 9 and BNBD 3 are chemotactic for immature bovine dendritic cells. *Vet Immunol Immunopathol* **143**, 87-107.
390. Pontarollo, R.A., Babiuk, L.A., Hecker, R., Van Drunen Littel-Van Den Hurk, S. (2002) Augmentation of cellular immune responses to bovine herpesvirus-1 glycoprotein D by vaccination with CpG-enhanced plasmid vectors. *J Gen Virol* **83**, 2973-81.
391. Biragyn, A., Tani, K., Grimm, M.C., Weeks, S., Kwak, L.W. (1999) Genetic fusion of chemokines to a self tumor antigen induces protective, T-cell dependent antitumor immunity. *Nat Biotechnol* **17**, 253-8.
392. Tang, Y., Zhang, L., Yuan, J., Akbulut, H., Maynard, J., Linton, P.J., Deisseroth, A. (2004) Multistep process through which adenoviral vector vaccine overcomes anergy to tumor-associated antigens. *Blood* **104**, 2704-13.
393. Kwak, L.W., Biragyn, A. (2010) Defensin-Antigen Fusion Proteins. The Govt. of USA. as represented by the Secretary of the Dept. of Health and Human Services Office of Technology Transfer, National Institutes of Health, United States.
394. Tang, Y., Deisseroth, A. (2006) Methods for immunotherapy of cancer. United States.

395. Nakamura, Y., Gojobori, T., Ikemura, T. (2000) Codon usage tabulated from international DNA sequence databases: status for the year 2000. *Nucleic Acids Res* **28**, 292.
396. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-5.
397. Schagger, H., von Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* **166**, 368-79.
398. Yount, N.Y., Yuan, J., Tarver, A., Castro, T., Diamond, G., Tran, P.A., Levy, J.N., McCullough, C., Cullor, J.S., Bevins, C.L., Selsted, M.E. (1999) Cloning and expression of bovine neutrophil beta-defensins. Biosynthetic profile during neutrophilic maturation and localization of mature peptide to novel cytoplasmic dense granules. *J Biol Chem* **274**, 26249-58.
399. Manoj, S., Griebel, P.J., Babiuk, L.A., van Drunen Littel-van den Hurk, S. (2003) Targeting with bovine CD154 enhances humoral immune responses induced by a DNA vaccine in sheep. *J Immunol* **170**, 989-96.
400. Labiuk, S.L., Babiuk, L.A., van Drunen Littel-van den Hurk, S. (2009) Major tegument protein VP8 of bovine herpesvirus 1 is phosphorylated by viral US3 and cellular CK2 protein kinases. *J Gen Virol* **90**, 2829-39.
401. Babiuk, L.A., Wardley, R.C., Rouse, B.T. (1975) Defense mechanisms against bovine herpesvirus: relationship of virus-host cell events to susceptibility to antibody-complement cell lysis. *Infect Immun* **12**, 958-63.
402. Mapletoft, J.W., Oumouna, M., Kovacs-Nolan, J., Latimer, L., Mutwiri, G., Babiuk, L.A., van Drunen Littel-van den Hurk, S. (2008) Intranasal immunization of mice with a formalin-inactivated bovine respiratory syncytial virus vaccine co-formulated with CpG oligodeoxynucleotides and polyphosphazenes results in enhanced protection. *J Gen Virol* **89**, 250-60.
403. Liang, R., van den Hurk, J.V., Landi, A., Lawman, Z., Deregt, D., Townsend, H., Babiuk, L.A., van Drunen Littel-van den Hurk, S. (2008) DNA prime protein boost strategies protect cattle from bovine viral diarrhea virus type 2 challenge. *J Gen Virol* **89**, 453-66.
404. Raggo, C., Habermehl, M., Babiuk, L.A., Griebel, P. (2000) The in vivo effects of recombinant bovine herpesvirus-1 expressing bovine interferon-gamma. *J Gen Virol* **81**, 2665-73.
405. Nakamoto, Y., Kaneko, S. (2006) Analysis of intrahepatic lymphocyte subsets in a transgenic mouse model of immune-mediated hepatocarcinogenesis. *J Exp Clin Cancer Res* **25**, 55-7.
406. Ghanekar, S.A., Nomura, L.E., Suni, M.A., Picker, L.J., Maecker, H.T., Maino, V.C. (2001) Gamma interferon expression in CD8(+) T cells is a marker for circulating cytotoxic T lymphocytes that recognize an HLA A2-restricted epitope of human cytomegalovirus phosphoprotein pp65. *Clin Diagn Lab Immunol* **8**, 628-31.
407. Jacobson, M.A., Maecker, H.T., Orr, P.L., D'Amico, R., Van Natta, M., Li, X.D., Pollard, R.B., Bredt, B.M. (2004) Results of a cytomegalovirus (CMV)-specific CD8+/interferon-gamma+ cytokine flow cytometry assay correlate with clinical evidence of protective immunity in patients with AIDS with CMV retinitis. *J Infect Dis* **189**, 1362-73.

408. Peng, S., Monie, A., Pang, X., Hung, C.F., Wu, T.C. (2011) Vascular disrupting agent DMXAA enhances the antitumor effects generated by therapeutic HPV DNA vaccines. *J Biomed Sci* **18**, 21.
409. Zhang, W., Lin, Y., Bai, Y., Tong, T., Wang, Q., Liu, N., Liu, G., Xiao, Y., Yang, T., Bu, Z., Tong, G., Wu, D. (2011) Identification of CD8+ cytotoxic T lymphocyte epitopes from porcine reproductive and respiratory syndrome virus matrix protein in BALB/c mice. *Virol J* **8**, 263.
410. Horton, H., Russell, N., Moore, E., Frank, I., Baydo, R., Havenar-Daughton, C., Lee, D., Deers, M., Hudgens, M., Weinhold, K., McElrath, M.J. (2004) Correlation between interferon- gamma secretion and cytotoxicity, in virus-specific memory T cells. *J Infect Dis* **190**, 1692-6.
411. Betts, M.R., Brenchley, J.M., Price, D.A., De Rosa, S.C., Douek, D.C., Roederer, M., Koup, R.A. (2003) Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. *J Immunol Methods* **281**, 65-78.
412. Badovinac, V.P., Harty, J.T. (2000) Intracellular staining for TNF and IFN-gamma detects different frequencies of antigen-specific CD8(+) T cells. *J Immunol Methods* **238**, 107-17.
413. Quade, M.J., Roth, J.A. (1999) Antigen-specific in vitro activation of T-lymphocyte subsets of cattle immunized with a modified live bovine herpesvirus 1 vaccine. *Viral Immunol* **12**, 9-21.
414. Endsley, J.J., Quade, M.J., Terhaar, B., Roth, J.A. (2002) BHV-1-Specific CD4+, CD8+, and gammadelta T cells in calves vaccinated with one dose of a modified live BHV-1 vaccine. *Viral Immunol* **15**, 385-93.
415. Platt, R., Burdett, W., Roth, J.A. (2006) Induction of antigen-specific T-cell subset activation to bovine respiratory disease viruses by a modified-live virus vaccine. *Am J Vet Res* **67**, 1179-84.
416. Platt, R., Roth, J.A., Royer, R.L., Thoen, C.O. (2006) Monitoring responses by use of five-color flow cytometry in subsets of peripheral T cells obtained from cattle inoculated with a killed Mycobacterium avium subsp paratuberculosis vaccine. *Am J Vet Res* **67**, 2050-8.
417. Gaddum, R.M., Cook, R.S., Furze, J.M., Ellis, S.A., Taylor, G. (2003) Recognition of bovine respiratory syncytial virus proteins by bovine CD8+ T lymphocytes. *Immunology* **108**, 220-9.
418. Schinnerling, K., Moos, V., Geelhaar, A., Allers, K., Loddenkemper, C., Friebel, J., Conrad, K., Kuhl, A.A., Erben, U., Schneider, T. (2011) Regulatory T cells in patients with Whipple's disease. *J Immunol* **187**, 4061-7.
419. Vremec, D., Zorbas, M., Scollay, R., Saunders, D.J., Ardavin, C.F., Wu, L., Shortman, K. (1992) The surface phenotype of dendritic cells purified from mouse thymus and spleen: investigation of the CD8 expression by a subpopulation of dendritic cells. *J Exp Med* **176**, 47-58.
420. Braun, R., Babiuk, L.A., van Drunen Littel-van den, H. (1998) Compatibility of plasmids expressing different antigens in a single DNA vaccine formulation. *J Gen Virol* **79 (Pt 12)**, 2965-70.
421. Sedegah, M., Charoenvit, Y., Minh, L., Belmonte, M., Majam, V.F., Abot, S., Ganeshan, H., Kumar, S., Bacon, D.J., Stowers, A., Narum, D.L., Carucci, D.J., Rogers, W.O.

(2004) Reduced immunogenicity of DNA vaccine plasmids in mixtures. *Gene Ther* **11**, 448-56.
422. Sedegah, M., Charoenvit, Y., Aguiar, J., Sacci, J., Hedstrom, R., Kumar, S., Belmonte, A., Lanar, D.E., Jones, T.R., Abot, E., Druilhe, P., Corradin, G., Epstein, J.E., Richie, T.L., Carucci, D.J., Hoffman, S.L. (2004) Effect on antibody and T-cell responses of mixing five GMP-produced DNA plasmids and administration with plasmid expressing GM-CSF. *Genes Immun* **5**, 553-61.
423. Ohteki, T., Fukao, T., Suzue, K., Maki, C., Ito, M., Nakamura, M., Koyasu, S. (1999) Interleukin 12-dependent interferon gamma production by CD8alpha+ lymphoid dendritic cells. *J Exp Med* **189**, 1981-6.
424. Dunne, P.J., Moran, B., Cummins, R.C., Mills, K.H. (2009) CD11c+CD8alpha+ dendritic cells promote protective immunity to respiratory infection with Bordetella pertussis. *J Immunol* **183**, 400-10.
425. Smith, C.M., Belz, G.T., Wilson, N.S., Villadangos, J.A., Shortman, K., Carbone, F.R., Heath, W.R. (2003) Cutting edge: conventional CD8 alpha+ dendritic cells are preferentially involved in CTL priming after footpad infection with herpes simplex virus-1. *J Immunol* **170**, 4437-40.
426. den Haan, J.M., Lehar, S.M., Bevan, M.J. (2000) CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. *J Exp Med* **192**, 1685-96.
427. Behboudi, S., Moore, A., Hill, A.V. (2004) Splenic dendritic cell subsets prime and boost CD8 T cells and are involved in the generation of effector CD8 T cells. *Cell Immunol* **228**, 15-9.
428. Maldonado-Lopez, R., De Smedt, T., Pajak, B., Heirman, C., Thielemans, K., Leo, O., Urbain, J., Maliszewski, C.R., Moser, M. (1999) Role of CD8alpha+ and CD8alpha- dendritic cells in the induction of primary immune responses in vivo. *J Leukoc Biol* **66**, 242-6.
429. Maldonado-Lopez, R., De Smedt, T., Michel, P., Godfroid, J., Pajak, B., Heirman, C., Thielemans, K., Leo, O., Urbain, J., Moser, M. (1999) CD8alpha+ and CD8alpha- subclasses of dendritic cells direct the development of distinct T helper cells in vivo. *J Exp Med* **189**, 587-92.
430. Suss, G., Shortman, K. (1996) A subclass of dendritic cells kills CD4 T cells via Fas/Fas-ligand-induced apoptosis. *J Exp Med* **183**, 1789-96.
431. Sin, J.I., Kim, J.J., Arnold, R.L., Shroff, K.E., McCallus, D., Pachuk, C., McElhiney, S.P., Wolf, M.W., Pompa-de Bruin, S.J., Higgins, T.J., Ciccarelli, R.B., Weiner, D.B. (1999) IL-12 gene as a DNA vaccine adjuvant in a herpes mouse model: IL-12 enhances Th1-type CD4+ T cell-mediated protective immunity against herpes simplex virus-2 challenge. *J Immunol* **162**, 2912-21.
432. Clerici, M., Shearer, G.M. (1994) The Th1-Th2 hypothesis of HIV infection: new insights. *Immunol Today* **15**, 575-81.
433. Kidd, P. (2003) Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. *Altern Med Rev* **8**, 223-46.
434. Agrawal, L., Haq, W., Hanson, C.V., Rao, D.N. (2003) Generating neutralizing antibodies, Th1 response and MHC non restricted immunogenicity of HIV-I env and gag peptides in liposomes and ISCOMs with in-built adjuvanticity. *J Immune Based Ther Vaccines* **1**, 5.

435. Mascarell, L., Fayolle, C., Bauche, C., Ladant, D., Leclerc, C. (2005) Induction of neutralizing antibodies and Th1-polarized and CD4-independent CD8+ T-cell responses following delivery of human immunodeficiency virus type 1 Tat protein by recombinant adenylate cyclase of Bordetella pertussis. *J Virol* **79**, 9872-84.
436. Bielinska, A.U., Janczak, K.W., Landers, J.J., Markovitz, D.M., Montefiori, D.C., Baker, J.R., Jr. (2008) Nasal immunization with a recombinant HIV gp120 and nanoemulsion adjuvant produces Th1 polarized responses and neutralizing antibodies to primary HIV type 1 isolates. *AIDS Res Hum Retroviruses* **24**, 271-81.
437. Liu, L., Wan, Y., Wu, L., Sun, J., Li, H., Ma, L., Shao, Y. (2010) Broader HIV-1 neutralizing antibody responses induced by envelope glycoprotein mutants based on the EIAV attenuated vaccine. *Retrovirology* **7**, 71.
438. McCluskie, M.J., Brazolot Millan, C.L., Gramzinski, R.A., Robinson, H.L., Santoro, J.C., Fuller, J.T., Widera, G., Haynes, J.R., Purcell, R.H., Davis, H.L. (1999) Route and method of delivery of DNA vaccine influence immune responses in mice and non-human primates. *Mol Med* **5**, 287-300.
439. Woolums, A.R., Siger, L., Johnson, S., Gallo, G., Conlon, J. (2003) Rapid onset of protection following vaccination of calves with multivalent vaccines containing modified-live or modified-live and killed BHV-1 is associated with virus-specific interferon gamma production. *Vaccine* **21**, 1158-64.
440. Ioannou, X.P., Griebel, P., Hecker, R., Babiuk, L.A., van Drunen Littel-van den Hurk, S. (2002) The immunogenicity and protective efficacy of bovine herpesvirus 1 glycoprotein D plus Emulsigen are increased by formulation with CpG oligodeoxynucleotides. *J Virol* **76**, 9002-10.
441. Rouse, B.T., Kaistha, S.D. (2006) A tale of 2 alpha-herpesviruses: lessons for vaccinologists. *Clin Infect Dis* **42**, 810-7.
442. Jones, C. (1998) Alphaherpesvirus latency: its role in disease and survival of the virus in nature. *Adv Virus Res* **51**, 81-133.
443. Weiner, D.B., Kennedy, R.C. (1999) Genetic vaccines. *Sci Am* **281**, 50-7.
444. Ott, G., Singh, M., Kazzaz, J., Briones, M., Soenawan, E., Ugozzoli, M., O'Hagan, D.T. (2002) A cationic sub-micron emulsion (MF59/DOTAP) is an effective delivery system for DNA vaccines. *J Control Release* **79**, 1-5.
445. Michel, M.L., Davis, H.L., Schleef, M., Mancini, M., Tiollais, P., Whalen, R.G. (1995) DNA-mediated immunization to the hepatitis B surface antigen in mice: aspects of the humoral response mimic hepatitis B viral infection in humans. *Proc Natl Acad Sci U S A* **92**, 5307-11.
446. Davis, H.L. (1997) Plasmid DNA expression systems for the purpose of immunization. *Curr Opin Biotechnol* **8**, 635-46.
447. Chattergoon, M.A., Robinson, T.M., Boyer, J.D., Weiner, D.B. (1998) Specific immune induction following DNA-based immunization through in vivo transfection and activation of macrophages/antigen-presenting cells. *J Immunol* **160**, 5707-18.
448. Sin, J.I., Kim, J.J., Ugen, K.E., Ciccarelli, R.B., Higgins, T.J., Weiner, D.B. (1998) Enhancement of protective humoral (Th2) and cell-mediated (Th1) immune responses against herpes simplex virus-2 through co-delivery of granulocyte-macrophage colony-stimulating factor expression cassettes. *Eur J Immunol* **28**, 3530-40.
449. Perrie, Y., Frederik, P.M., Gregoriadis, G. (2001) Liposome-mediated DNA vaccination: the effect of vesicle composition. *Vaccine* **19**, 3301-10.

450. Commander, N.J., Brewer, J.M., Wren, B.W., Spencer, S.A., Macmillan, A.P., Stack, J.A. (2010) Liposomal delivery of p-ialB and p-omp25 DNA vaccines improves immunogenicity but fails to provide full protection against B. melitensis challenge. *Genet Vaccines Ther* **8**, 5.
451. Greenland, J.R., Letvin, N.L. (2007) Chemical adjuvants for plasmid DNA vaccines. *Vaccine* **25**, 3731-41.
452. Mackenzie-Dyck, S., Kovacs-Nolan, J., Snider, M., Babiuk, L.A., van Drunen Littel-van den Hurk, S. (2014) Inclusion of the bovine neutrophil beta-defensin 3 with glycoprotein D of Bovine herpesvirus-1 in a DNA vaccine modulates immune responses of mice and cattle. *Clin Vaccine Immunol* **21**, 463-477.
453. Riedl, P., Reimann, J., Schirmbeck, R. (2006) Complexes of DNA vaccines with cationic, antigenic peptides are potent, polyvalent CD8(+) T-cell-stimulating immunogens. *Methods Mol Med* **127**, 159-69.
454. Riedl, P., Reimann, J., Schirmbeck, R. (2004) Peptides containing antigenic and cationic domains have enhanced, multivalent immunogenicity when bound to DNA vaccines. *J Mol Med* **82**, 144-52.
455. Singh, M., Briones, M., Ott, G., O'Hagan, D. (2000) Cationic microparticles: A potent delivery system for DNA vaccines. *Proc Natl Acad Sci U S A* **97**, 811-6.
456. Rouse, R.J., Nair, S.K., Lydy, S.L., Bowen, J.C., Rouse, B.T. (1994) Induction in vitro of primary cytotoxic T-lymphocyte responses with DNA encoding herpes simplex virus proteins. *J Virol* **68**, 5685-9.
457. Sin, J.I., Ayyavoo, V., Boyer, J., Kim, J., Ciccarelli, R.B., Weiner, D.B. (1999) Protective immune correlates can segregate by vaccine type in a murine herpes model system. *Int Immunol* **11**, 1763-73.
458. Sin, J.I., Kim, J.J., Boyer, J.D., Ciccarelli, R.B., Higgins, T.J., Weiner, D.B. (1999) In vivo modulation of vaccine-induced immune responses toward a Th1 phenotype increases potency and vaccine effectiveness in a herpes simplex virus type 2 mouse model. *J Virol* **73**, 501-9.
459. Yu, H., Babiuk, L.A., van Drunen Littel-van den Hurk, S. (2008) Strategies for loading dendritic cells with hepatitis C NS5a antigen and inducing protective immunity. *J Viral Hepat* **15**, 459-70.
460. Liu, Y.D., Goetze, A.M., Bass, R.B., Flynn, G.C. (2011) N-terminal glutamate to pyroglutamate conversion in vivo for human IgG2 antibodies. *J Biol Chem* **286**, 11211-7.
461. Yu, H., Huang, H., Xiang, J., Babiuk, L.A., van Drunen Littel-van den Hurk, S. (2006) Dendritic cells pulsed with hepatitis C virus NS3 protein induce immune responses and protection from infection with recombinant vaccinia virus expressing NS3. *J Gen Virol* **87**, 1-10.
462. Wiesel, M., Walton, S., Richter, K., Oxenius, A. (2009) Virus-specific CD8 T cells: activation, differentiation and memory formation. *APMIS* **117**, 356-81.
463. Favoreel, H.W., Van Minnebruggen, G., Van de Walle, G.R., Ficinska, J., Nauwynck, H.J. (2006) Herpesvirus interference with virus-specific antibodies: bridging antibodies, internalizing antibodies, and hiding from antibodies. *Vet Microbiol* **113**, 257-63.
464. Hewitt, E.W. (2003) The MHC class I antigen presentation pathway: strategies for viral immune evasion. *Immunology* **110**, 163-9.

465. Wei, H., Wang, Y., Chowdhury, S.I. (2011) Bovine herpesvirus type 1 (BHV-1) UL49.5 luminal domain residues 30 to 32 are critical for MHC-I down-regulation in virus-infected cells. *PLoS One* **6**, e25742.
466. Gao, G.F., Jakobsen, B.K. (2000) Molecular interactions of coreceptor CD8 and MHC class I: the molecular basis for functional coordination with the T-cell receptor. *Immunol Today* **21**, 630-6.
467. Wang, B., Norbury, C.C., Greenwood, R., Bennink, J.R., Yewdell, J.W., Frelinger, J.A. (2001) Multiple paths for activation of naive CD8+ T cells: CD4-independent help. *J Immunol* **167**, 1283-9.
468. Manoj, S., Babiuk, L.A., van Drunen Littel-van den Hurk, S. (2003) Immunization with a dicistronic plasmid expressing a truncated form of bovine herpesvirus-1 glycoprotein D and the amino-terminal subunit of glycoprotein B results in reduced gB-specific immune responses. *Virology* **313**, 296-307.
469. Kawabata, K., Takakura, Y., Hashida, M. (1995) The fate of plasmid DNA after intravenous injection in mice: involvement of scavenger receptors in its hepatic uptake. *Pharm Res* **12**, 825-30.
470. Martin, M.E., Rice, K.G. (2007) Peptide-guided gene delivery. *AAPS J* **9**, E18-29.
471. Gerashchenko, O.L., Zhuravel, E.V., Skachkova, O.V., Khranovska, N.N., Filonenko, V.V., Pogrebnoy, P.V., Soldatkina, M.A. (2013) Biologic activities of recombinant human-beta-defensin-4 toward cultured human cancer cells. *Exp Oncol* **35**, 76-82.
472. Licciardi, M., Campisi, M., Cavallaro, G., Cervello, M., Azzolina, A., Giammona, G. (2006) Synthesis and characterization of polyaminoacidic polycations for gene delivery. *Biomaterials* **27**, 2066-75.
473. Lehto, T., Simonson, O.E., Mager, I., Ezzat, K., Sork, H., Copolovici, D.M., Viola, J.R., Zaghloul, E.M., Lundin, P., Moreno, P.M., Mae, M., Oskolkov, N., Suhorutsenko, J., Smith, C.I., Andaloussi, S.E. (2011) A peptide-based vector for efficient gene transfer in vitro and in vivo. *Mol Ther* **19**, 1457-67.
474. Wattiaux, R., Laurent, N., Wattiaux-De Coninck, S., Jadot, M. (2000) Endosomes, lysosomes: their implication in gene transfer. *Adv Drug Deliv Rev* **41**, 201-8.
475. Zelphati, O., Szoka, F.C., Jr. (1996) Mechanism of oligonucleotide release from cationic liposomes. *Proc Natl Acad Sci U S A* **93**, 11493-8.
476. Pedone, E., Cavallaro, G., Richardson, S.C., Duncan, R., Giammona, G. (2001) alpha,beta-poly(asparthylhydrazide)-glycidyltrimethylammonium chloride copolymers (PAHy-GTA): novel polymers with potential for DNA delivery. *J Control Release* **77**, 139-53.
477. Lingnau, K., Riedl, K., von Gabain, A. (2007) IC31 and IC30, novel types of vaccine adjuvant based on peptide delivery systems. *Expert Rev Vaccines* **6**, 741-6.
478. Niidome, T., Takaji, K., Urakawa, M., Ohmori, N., Wada, A., Hirayama, T., Aoyagi, H. (1999) Chain length of cationic alpha-helical peptide sufficient for gene delivery into cells. *Bioconjug Chem* **10**, 773-80.
479. Haines, A.M., Irvine, A.S., Mountain, A., Charlesworth, J., Farrow, N.A., Husain, R.D., Hyde, H., Ketteringham, H., McDermott, R.H., Mulcahy, A.F., Mustoe, T.L., Reid, S.C., Rouquette, M., Shaw, J.C., Thatcher, D.R., Welsh, J.H., Williams, D.E., Zauner, W., Phillips, R.O. (2001) CL22 - a novel cationic peptide for efficient transfection of mammalian cells. *Gene Ther* **8**, 99-110.

480. Foged, C., Arigita, C., Sundblad, A., Jiskoot, W., Storm, G., Frokjaer, S. (2004) Interaction of dendritic cells with antigen-containing liposomes: effect of bilayer composition. *Vaccine* **22**, 1903-13.
481. Wyman, T.B., Nicol, F., Zelphati, O., Scaria, P.V., Plank, C., Szoka, F.C., Jr. (1997) Design, synthesis, and characterization of a cationic peptide that binds to nucleic acids and permeabilizes bilayers. *Biochemistry* **36**, 3008-17.
482. Wadia, J.S., Stan, R.V., Dowdy, S.F. (2004) Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat Med* **10**, 310-5.
483. Heitz, F., Morris, M.C., Divita, G. (2009) Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics. *Br J Pharmacol* **157**, 195-206.
484. Conner, S.D., Schmid, S.L. (2003) Regulated portals of entry into the cell. *Nature* **422**, 37-44.
485. Zanta, M.A., Belguise-Valladier, P., Behr, J.P. (1999) Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus. *Proc Natl Acad Sci U S A* **96**, 91-6.
486. Sandgren, S., Wittrup, A., Cheng, F., Jonsson, M., Eklund, E., Busch, S., Belting, M. (2004) The human antimicrobial peptide LL-37 transfers extracellular DNA plasmid to the nuclear compartment of mammalian cells via lipid rafts and proteoglycan-dependent endocytosis. *J Biol Chem* **279**, 17951-6.
487. Labeur, M.S., Roters, B., Pers, B., Mehling, A., Luger, T.A., Schwarz, T., Grabbe, S. (1999) Generation of tumor immunity by bone marrow-derived dendritic cells correlates with dendritic cell maturation stage. *J Immunol* **162**, 168-75.
488. Rivas-Caicedo, A., Soldevila, G., Fortoul, T.I., Castell-Rodriguez, A., Flores-Romo, L., Garcia-Zepeda, E.A. (2009) Jak3 is involved in dendritic cell maturation and CCR7-dependent migration. *PLoS One* **4**, e7066.
489. Singh-Jasuja, H., Thiolat, A., Ribon, M., Boissier, M.C., Bessis, N., Rammensee, H.G., Decker, P. (2013) The mouse dendritic cell marker CD11c is down-regulated upon cell activation through Toll-like receptor triggering. *Immunobiology* **218**, 28-39.
490. Asano, Y., Komuro, T., Tada, T. (1990) Synergistic T-T cell interaction present in alloreactivity: determination of 'MLR helper' T cell subsets. *Int Immunol* **2**, 1203-11.
491. Peltola, V., Ruuskanen, O. (2008) Editorial commentary: Respiratory viral infections in developing countries: common, severe, and unrecognized. *Clin Infect Dis* **46**, 58-60.
492. Graham, D.A. (2013) Bovine herpes virus-1 (BoHV-1) in cattle-a review with emphasis on reproductive impacts and the emergence of infection in Ireland and the United Kingdom. *Ir Vet J* **66**, 15.
493. Hartel, H., Nikunen, S., Neuvonen, E., Tanskanen, R., Kivela, S.L., Aho, R., Soveri, T., Saloniemi, H. (2004) Viral and bacterial pathogens in bovine respiratory disease in Finland. *Acta Vet Scand* **45**, 193-200.
494. Deshpande, M.S., Ambagala, T.C., Hegde, N.R., Hariharan, M.J., Navaratnam, M., Srikumaran, S. (2002) Induction of cytotoxic T-lymphocytes specific for bovine herpesvirus-1 by DNA immunization. *Vaccine* **20**, 3744-51.
495. Whetstone, C.A., Miller, J.M., Seal, B.S., Bello, L.J., Lawrence, W.C. (1992) Latency and reactivation of a thymidine kinase-negative bovine herpesvirus 1 deletion mutant. *Arch Virol* **122**, 207-14.

496. Coban, C., Kobiyama, K., Jounai, N., Tozuka, M., Ishii, K.J. (2013) DNA vaccines: a simple DNA sensing matter? *Hum Vaccin Immunother* **9**, 2216-21.
497. Bins, A.D., Jorritsma, A., Wolkers, M.C., Hung, C.F., Wu, T.C., Schumacher, T.N., Haanen, J.B. (2005) A rapid and potent DNA vaccination strategy defined by in vivo monitoring of antigen expression. *Nat Med* **11**, 899-904.
498. Coban, C., Kobiyama, K., Aoshi, T., Takeshita, F., Horii, T., Akira, S., Ishii, K.J. Novel strategies to improve DNA vaccine immunogenicity. *Curr Gene Ther* **11**, 479-84.
499. Bajer, A.A., Garcia-Tapia, D., Jordan, K.R., Haas, K.M., Werling, D., Howard, C.J., Estes, D.M. (2003) Peripheral blood-derived bovine dendritic cells promote IgG1-restricted B cell responses in vitro. *J Leukoc Biol* **73**, 100-6.
500. Hope, J.C., Werling, D., Collins, R.A., Mertens, B., Howard, C.J. (2000) Flt-3 ligand, in combination with bovine granulocyte-macrophage colony-stimulating factor and interleukin-4, promotes the growth of bovine bone marrow derived dendritic cells. *Scand J Immunol* **51**, 60-6.
501. Howard, C.J., Hope, J.C. (2000) Dendritic cells, implications on function from studies of the afferent lymph veiled cell. *Vet Immunol Immunopathol* **77**, 1-13.
502. Lin, S.L., Chien, C.W., Han, C.L., Chen, E.S., Kao, S.H., Chen, Y.J., Liao, F. (2010) Temporal proteomics profiling of lipid rafts in CCR6-activated T cells reveals the integration of actin cytoskeleton dynamics. *J Proteome Res* **9**, 283-97.
503. Oppenheim, J.J., Yang, D., Biragyn, A., Howard, O.M., Plotz, P. (2002) Chemokine receptors on dendritic cells promote autoimmune reactions. *Arthritis Res* **4 Suppl 3**, S183-8.
504. Reid, E., Juleff, N., Gubbins, S., Prentice, H., Seago, J., Charleston, B. (2011) Bovine plasmacytoid dendritic cells are the major source of type I interferon in response to foot-and-mouth disease virus in vitro and in vivo. *J Virol* **85**, 4297-308.
505. Gibson, A., Miah, S., Griebel, P., Brownlie, J., Werling, D. (2012) Identification of a lineage negative cell population in bovine peripheral blood with the ability to mount a strong type I interferon response. *Dev Comp Immunol* **36**, 332-41.
506. Grassi, F., Dezutter-Dambuyant, C., McIlroy, D., Jacquet, C., Yoneda, K., Imamura, S., Boumsell, L., Schmitt, D., Autran, B., Debre, P., Hosmalin, A. (1998) Monocyte-derived dendritic cells have a phenotype comparable to that of dermal dendritic cells and display ultrastructural granules distinct from Birbeck granules. *J Leukoc Biol* **64**, 484-93.
507. He, Y., Zhang, J., Donahue, C., Falo, L.D., Jr. (2006) Skin-derived dendritic cells induce potent CD8(+) T cell immunity in recombinant lentivector-mediated genetic immunization. *Immunity* **24**, 643-56.
508. Allenspach, E.J., Lemos, M.P., Porrett, P.M., Turka, L.A., Laufer, T.M. (2008) Migratory and lymphoid-resident dendritic cells cooperate to efficiently prime naive CD4 T cells. *Immunity* **29**, 795-806.
509. Mount, A.M., Smith, C.M., Kupresanin, F., Stoermer, K., Heath, W.R., Belz, G.T. (2008) Multiple dendritic cell populations activate CD4+ T cells after viral stimulation. *PLoS One* **3**, e1691.
510. Harada, S., Kimura, T., Fujiki, H., Nakagawa, H., Ueda, Y., Itoh, T., Yamagishi, H., Sonoda, Y. (2007) Flt3 ligand promotes myeloid dendritic cell differentiation of human hematopoietic progenitor cells: possible application for cancer immunotherapy. *Int J Oncol* **30**, 1461-8.

511. Miller, G., Pillarisetty, V.G., Shah, A.B., Lahrs, S., DeMatteo, R.P. (2003) Murine Flt3 ligand expands distinct dendritic cells with both tolerogenic and immunogenic properties. *J Immunol* **170**, 3554-64.
512. Huang, Y., Babiuk, L.A., van Drunen Littel-van den Hurk, S. (2006) The cell-mediated immune response induced by plasmid encoding bovine herpesvirus 1 glycoprotein B is enhanced by plasmid encoding IL-12 when delivered intramuscularly or by gene gun, but not after intradermal injection. *Vaccine* **24**, 5349-59.
513. Kemnade, J.O., Seethammagari, M., Narayanan, P., Levitt, J.M., McCormick, A.A., Spencer, D.M. (2012) Off-the-shelf adenoviral-mediated immunotherapy via bicistronic expression of tumor antigen and iMyD88/CD40 adjuvant. *Mol Ther* **20**, 1462-71.
514. Stiles, K.M., Whitbeck, J.C., Lou, H., Cohen, G.H., Eisenberg, R.J., Krummenacher, C. (2010) Herpes simplex virus glycoprotein D interferes with binding of herpesvirus entry mediator to its ligands through downregulation and direct competition. *J Virol* **84**, 11646-60.
515. Mei, H.F., Jin, X.B., Zhu, J.Y., Zeng, A.H., Wu, Q., Lu, X.M., Li, X.B., Shen, J. (2012) beta-defensin 2 as an adjuvant promotes anti-melanoma immune responses and inhibits the growth of implanted murine melanoma in vivo. *PLoS One* **7**, e31328.
516. Idoyaga, J., Lubkin, A., Fiorese, C., Lahoud, M.H., Caminschi, I., Huang, Y., Rodriguez, A., Clausen, B.E., Park, C.G., Trumpfheller, C., Steinman, R.M. (2011) Comparable T helper 1 (Th1) and CD8 T-cell immunity by targeting HIV gag p24 to CD8 dendritic cells within antibodies to Langerin, DEC205, and Clec9A. *Proc Natl Acad Sci U S A* **108**, 2384-9.
517. Bozzacco, L., Trumpfheller, C., Siegal, F.P., Mehandru, S., Markowitz, M., Carrington, M., Nussenzweig, M.C., Piperno, A.G., Steinman, R.M. (2007) DEC-205 receptor on dendritic cells mediates presentation of HIV gag protein to CD8+ T cells in a spectrum of human MHC I haplotypes. *Proc Natl Acad Sci U S A* **104**, 1289-94.
518. Kronin, V., Fitzmaurice, C.J., Caminschi, I., Shortman, K., Jackson, D.C., Brown, L.E. (2001) Differential effect of CD8(+) and CD8(-) dendritic cells in the stimulation of secondary CD4(+) T cells. *Int Immunol* **13**, 465-73.
519. Parker, D.C. (1993) T cell-dependent B cell activation. *Annu Rev Immunol* **11**, 331-60.
520. Mozdzanowska, K., Furchner, M., Zharikova, D., Feng, J., Gerhard, W. (2005) Roles of CD4+ T-cell-independent and -dependent antibody responses in the control of influenza virus infection: evidence for noncognate CD4+ T-cell activities that enhance the therapeutic activity of antiviral antibodies. *J Virol* **79**, 5943-51.
521. Otten, G.R., Doe, B., Schaefer, M., Chen, M., Selby, M.J., Goldbeck, C., Hong, M., Xu, F., Ulmer, J.B. (2000) Relative potency of cellular and humoral immune responses induced by DNA vaccination. *Intervirology* **43**, 227-32.
522. Melroe, G.T., DeLuca, N.A., Knipe, D.M. (2004) Herpes simplex virus 1 has multiple mechanisms for blocking virus-induced interferon production. *J Virol* **78**, 8411-20.
523. Xing, J., Ni, L., Wang, S., Wang, K., Lin, R., Zheng, C. (2013) Herpes simplex virus 1-encoded tegument protein VP16 abrogates the production of beta interferon (IFN) by inhibiting NF-kappaB activation and blocking IFN regulatory factor 3 to recruit its coactivator CBP. *J Virol* **87**, 9788-801.
524. Saira, K., Zhou, Y., Jones, C. (2007) The infected cell protein 0 encoded by bovine herpesvirus 1 (bICP0) induces degradation of interferon response factor 3 and, consequently, inhibits beta interferon promoter activity. *J Virol* **81**, 3077-86.

525. Dai, Y., Zhu, Y., Harn, D.A., Wang, X., Tang, J., Zhao, S., Lu, F., Guan, X. (2009) DNA vaccination by electroporation and boosting with recombinant proteins enhances the efficacy of DNA vaccines for Schistosomiasis japonica. *Clin Vaccine Immunol* **16**, 1796-803.
526. Hughes, G., Babiuk, L.A., van Drunen Littel-van den Hurk, S. (1988) Functional and topographical analyses of epitopes on bovine herpesvirus type 1 glycoprotein IV. *Arch Virol* **103**, 47-60.
527. Xu, N., Wang, Y.S., Pan, W.B., Xiao, B., Wen, Y.J., Chen, X.C., Chen, L.J., Deng, H.X., You, J., Kan, B., Fu, A.F., Li, D., Zhao, X., Wei, Y.Q. (2008) Human alpha-defensin-1 inhibits growth of human lung adenocarcinoma xenograft in nude mice. *Mol Cancer Ther* **7**, 1588-97.
528. Riedl, S., Zweytick, D., Lohner, K. (2011) Membrane-active host defense peptides--challenges and perspectives for the development of novel anticancer drugs. *Chem Phys Lipids* **164**, 766-81.
529. Kisich, K.O., Heifets, L., Higgins, M., Diamond, G. (2001) Antimycobacterial agent based on mRNA encoding human beta-defensin 2 enables primary macrophages to restrict growth of Mycobacterium tuberculosis. *Infect Immun* **69**, 2692-9.
530. Alberts, B., Jonhnson, A., Lewis, J., al., e. (2002) Helper T cells and Lymphocyte Activation. In *Molecular Biology of the Cell*Garland Science, New York.
531. Amakata, Y., Fujiyama, Y., Andoh, A., Hodohara, K., Bamba, T. (2001) Mechanism of NK cell activation induced by coculture with dendritic cells derived from peripheral blood monocytes. *Clin Exp Immunol* **124**, 214-22.
532. Chalifour, A., Jeannin, P., Gauchat, J.F., Blaecke, A., Malissard, M., N'Guyen, T., Thieblemont, N., Delneste, Y. (2004) Direct bacterial protein PAMP recognition by human NK cells involves TLRs and triggers alpha-defensin production. *Blood* **104**, 1778-83.
533. Michel, T., Poli, A., Domingues, O., Mauffray, M., Theresine, M., Brons, N.H., Hentges, F., Zimmer, J. (2012) Mouse lung and spleen natural killer cells have phenotypic and functional differences, in part influenced by macrophages. *PLoS One* **7**, e51230.
534. Adib-Conquy, M., Scott-Algara, D., Cavaillon, J.M., Souza-Fonseca-Guimaraes, F. (2014) TLR-mediated activation of NK cells and their role in bacterial/viral immune responses in mammals. *Immunol Cell Biol* **92**, 256-62.
535. Koh, C.Y., Yuan, D. (2000) The functional relevance of NK-cell-mediated upregulation of antigen-specific IgG2a responses. *Cell Immunol* **204**, 135-42.
536. Cruse, J.M., Lewis, R.E., Wang, H. (2004) Immunology Guidebook. Elsevier Academic Press, San Diego 516.
537. Bates, E.E., Fournier, N., Garcia, E., Valladeau, J., Durand, I., Pin, J.J., Zurawski, S.M., Patel, S., Abrams, J.S., Lebecque, S., Garrone, P., Saeland, S. (1999) APCs express DCIR, a novel C-type lectin surface receptor containing an immunoreceptor tyrosine-based inhibitory motif. *J Immunol* **163**, 1973-83.
538. Ariizumi, K., Shen, G.L., Shikano, S., Ritter, R., 3rd, Zukas, P., Edelbaum, D., Morita, A., Takashima, A. (2000) Cloning of a second dendritic cell-associated C-type lectin (dectin-2) and its alternatively spliced isoforms. *J Biol Chem* **275**, 11957-63.
539. Ariizumi, K., Shen, G.L., Shikano, S., Xu, S., Ritter, R., 3rd, Kumamoto, T., Edelbaum, D., Morita, A., Bergstresser, P.R., Takashima, A. (2000) Identification of a novel,

dendritic cell-associated molecule, dectin-1, by subtractive cDNA cloning. *J Biol Chem* **275**, 20157-67.
540. Colonna, M., Samaridis, J., Angman, L. (2000) Molecular characterization of two novel C-type lectin-like receptors, one of which is selectively expressed in human dendritic cells. *Eur J Immunol* **30**, 697-704.
541. Sallusto, F., Lanzavecchia, A. (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med* **179**, 1109-18.
542. Jordens, R., Thompson, A., Amons, R., Koning, F. (1999) Human dendritic cells shed a functional, soluble form of the mannose receptor. *Int Immunol* **11**, 1775-80.
543. Regnault, A., Lankar, D., Lacabanne, V., Rodriguez, A., Thery, C., Rescigno, M., Saito, T., Verbeek, S., Bonnerot, C., Ricciardi-Castagnoli, P., Amigorena, S. (1999) Fcgamma receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. *J Exp Med* **189**, 371-80.
544. Maurer, D., Fiebiger, E., Reininger, B., Ebner, C., Petzelbauer, P., Shi, G.P., Chapman, H.A., Stingl, G. (1998) Fc epsilon receptor I on dendritic cells delivers IgE-bound multivalent antigens into a cathepsin S-dependent pathway of MHC class II presentation. *J Immunol* **161**, 2731-9.
545. Bates, E.E., Dieu, M.C., Ravel, O., Zurawski, S.M., Patel, S., Bridon, J.M., Ait-Yahia, S., Vega, F., Jr., Banchereau, J., Lebecque, S. (1998) CD40L activation of dendritic cells down-regulates DORA, a novel member of the immunoglobulin superfamily. *Mol Immunol* **35**, 513-24.
546. Cella, M., Dohring, C., Samaridis, J., Dessing, M., Brockhaus, M., Lanzavecchia, A., Colonna, M. (1997) A novel inhibitory receptor (ILT3) expressed on monocytes, macrophages, and dendritic cells involved in antigen processing. *J Exp Med* **185**, 1743-51.
547. Colaco, C.A. (1998) Towards a unified theory of immunity: dendritic cells, stress proteins and antigen capture. *Cell Mol Biol (Noisy-le-grand)* **44**, 883-90.
548. Hertz, C.J., Kiertscher, S.M., Godowski, P.J., Bouis, D.A., Norgard, M.V., Roth, M.D., Modlin, R.L. (2001) Microbial lipopeptides stimulate dendritic cell maturation via Toll-like receptor 2. *J Immunol* **166**, 2444-50.
549. Mosialos, G., Birkenbach, M., Ayehunie, S., Matsumura, F., Pinkus, G.S., Kieff, E., Langhoff, E. (1996) Circulating human dendritic cells differentially express high levels of a 55-kd actin-bundling protein. *Am J Pathol* **148**, 593-600.
550. Nobes, C., Marsh, M. (2000) Dendritic cells: new roles for Cdc42 and Rac in antigen uptake? *Curr Biol* **10**, R739-41.
551. Garrett, W.S., Chen, L.M., Kroschewski, R., Ebersold, M., Turley, S., Trombetta, S., Galan, J.E., Mellman, I. (2000) Developmental control of endocytosis in dendritic cells by Cdc42. *Cell* **102**, 325-34.
552. West, M.A., Prescott, A.R., Eskelinen, E.L., Ridley, A.J., Watts, C. (2000) Rac is required for constitutive macropinocytosis by dendritic cells but does not control its downregulation. *Curr Biol* **10**, 839-48.
553. Bates, E.E., Ravel, O., Dieu, M.C., Ho, S., Guret, C., Bridon, J.M., Ait-Yahia, S., Briere, F., Caux, C., Banchereau, J., Lebecque, S. (1997) Identification and analysis of a novel

member of the ubiquitin family expressed in dendritic cells and mature B cells. *Eur J Immunol* **27**, 2471-7.